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BIOCHEMISTRY OF THE USTILAGINALES

VII. ANTIBIOTIC ACTIVITY OF USTILAGIC ACID[1]

BY R. H. HASKINS AND J. A. THORN

Abstract

The antibiotic activity of *Ustilago zeae* (PRL 119) cultures is associated mainly with the culture solids and it has been shown that a mixture of glucolipids, called ustilagic acid and produced in good yields by the fungus, is responsible for part, if not all, of the observed activity. The antibiotic activity of ustilagic acid corresponds to that originally attributed to ustizeain B and therefore these two are considered to be identical. All samples of ustilagic acid obtained showed antibiotic activity and it has not been possible by paper chromatography to separate an active principle from the acid. Recrystallization of ustilagic acid did not alter its antibiotic properties. Alkaline hydrolysis of the acid destroyed its activity. Heating the culture mixture for up to an hour at temperatures less than 100° C. did not alter materially the antibiotic properties of the mixture or of the acid extracted therefrom. The antifungal spectrum is broad and the antibacterial spectrum includes more Gram-positive than Gram-negative organisms. Preliminary tests with rats indicated that ustilagic acid is non-toxic when administered orally up to at least 5% of the diet, and with mice, orally up to at least 1% of the diet or intraperitoneally in amounts up to 1.5 gm. per kgm. of body weight.

Introduction

Since the production by *Ustilago zeae* (corn smut) of substances with antibiotic properties was first reported by Haskins (1, 2), considerable work has been done on the study of these properties and on the isolation of the active principles. Though agar plate techniques showed excellent inhibition zones surrounding growing smut colonies, the liquid from centrifuged or filtered culture mixtures, aerobically fermented by the smut fungus in submerged growth, possessed only low activity. Many strains and isolates of *Ustilago* were examined for antibiotic activity. Some showed greater activity than others, but, in all cases, the activity of the culture-mixture filtrate was disappointingly low. Certain strains showed increased antibacterial and antifungal activity, while others showed slight increases in one and not the other—an indication, perhaps, that more than one substance might be responsible for the observed activities.

[1] *Manuscript received July 20, 1951.*

Contribution from the National Research Council, Prairie Regional Laboratory, Saskatoon, Sask. Issued as Paper No. 125 on the Industrial Utilization of Wastes and Surpluses, and as N.R.C. No. 2530. Presented in part at the 119th Meeting of the American Chemical Society, Cleveland, Ohio, April 8–11, 1951.

[The October number of this Journal (Can. J. Botany, 29 : 451-584. 1951) was issued November 2, 1951.]

In attempts to isolate active material from culture filtrates, the adsorption on activated charcoal of the antibiotic substance and the extraction of the culture mixture with various solvents such as ether, chloroform, carbon tetrachloride, petroleum ether, carbon disulphide, amyl acetate, etc. resulted in only little extraction and concentration of an active principle. It was found that the antibiotic activity of the filtrates of various cultures was fairly constant, while the activity of the solids from these same cultures varied, and that the substance(s) responsible for the antibiosis were located mainly in the culture solids. A good part of the culture solids is a crystalline material which has been shown to be a mixture of partially acylated derivatives of a di-D-glucosyl-dihydroxyhexadecanoic acid (3, 4) and was named ustilagic acid. When tests showed this material to possess antibiotic activity similar to that of culture mixtures containing comparable amounts of ustilagic acid, it became evident that the substance responsible for the antibiotic activity was either ustilagic acid itself or a substance(s) with similar solubility properties. Pure white crystalline samples of extracted ustilagic acid showed excellent activity. Attempts to separate the antibiotic activities, formerly called the ustizeains, from ustilagic acid by means of solvent extraction and partition chromatography were unsuccessful. It thus became obvious that the ustilagic acid was responsible for most, if not all, of the antibiotic activity of smut cultures. The activity of ustilagic acid as such corresponds with the activity originally attributed to ustizeain B, therefore these two are considered to be one and the same substance.

For convenience, the substances responsible for antibacterial and antifungal activity originally were named ustilagin A, B, etc. (1). These names, though seemingly appropriate, were discarded in favor of ustizeain A, B, etc. (2) when it was learned that the name "ustilagin" or "ustilagine" had been applied much earlier (5) to alkaloidal materials isolated from heavily smutted cobs of corn. The name ustilagic acid will be used for the antibiotic substance until such time as other active materials may be shown to be present. Since ustilagic acid has been shown to be a mixture (4), it is possible that certain of the constituents may be more antibiotically active than others, but tests of this will have to await adequate separation of the constituents of the mixture.

Recrystallization of ustilagic acid from aqueous methanol did not change the antibiotic activity of the acid. Heating of the culture mixture prior to extraction of the ustilagic acid did not appear to affect the antibiotic activity of the culture mixture or of the ustilagic acid extracted therefrom. Hydrolysis of ustilagic acid with sodium hydroxide destroyed its antibiotic properties, indicating that the observed activity resides in the acid itself rather than in one or more of its various degradation products. Dilute aqueous solutions of the sodium salt have been prepared with some success and these had detectable antibiotic properties, though it was not known to what extent the acid had been hydrolyzed, or whether the observed activity was due to a sodium salt or to residual ustilagic acid.

The antifungal spectrum seems to be quite broad, and the antibacterial spectrum includes more Gram-positive than Gram-negative bacteria.

Preliminary tests with rats and with white mice indicated that ustilagic acid is at least relatively nontoxic when administered orally or intraperitoneally.

Methods

The culture mixtures used in this study were produced by the dissimilation of available cerelose in 5–10% cerelose nutrient medium (2) by *Ustilago zeae* PRL 119, growing in agitated submerged culture at 30° C. The fermentations were carried out either in half-liter flasks shaken for three–five days on a Gump rotary shaker, with a 1 in. radius of motion, operating at 225 r.p.m.; or in 30-liter stirred, aerated, stainless steel fermentors.

Since that time, when it was realized that the small amount of ustilagic acid in aqueous solution must be responsible for most, if not all, of the observed antibiotic effects of the organism, the activity of ustilagic acid as such has been studied. All of the ustilagic acid used in these studies, unless otherwise stated, was extracted from the culture solids with warm (approximately 50° C.) methanol, precipitated from the methanol by the addition of nine parts of warm (approximately 50° C.) water followed by slow cooling, recovered from the dilute aqueous methanol by filtration, and finally washed with ether. The large yields of ustilagic acid ranging from 5 to over 28 gm. per liter in present culture mixtures, the simplicity of the methods of extracting relatively pure crystalline material (2, 3, 6), and the method of chemically estimating ustilagic acid present in culture broths (6) have made the establishment of "units" of ustilagic acid unnecessary. Though a quantitative bioassay (as a means of measuring ustilagic acid) has not been necessary in these *in vitro* studies, such assays have been investigated.

In the development of a bioassay, the use of filter paper squares, blotter disks, porcelain "penicylinders", or metal penicylinders has proved unsatisfactory. The most repeatable assay results have been obtained using cylinders, approximately 7 mm. long, with ground ends, and cut from 10 mm. O.D. Pyrex glass tubing. In testing samples for activity, these cylinders were flamed sufficiently to enable them to stick to the surface of the Difco potato-dextrose or Sabouraud's dextrose agar plates on which they were placed. Half-milliliter aliquots of the samples to be tested were placed in the cylinders and the plates incubated for 24 hr. at 30° C., or at room temperature, as were the control plates, the cylinders of which contained known concentrations of ustilagic acid. The preliminary incubation is advisable to produce adequate inhibition zones. The surface of the agar was flooded then with a suspension of the test organism, or, with less trouble and more repeatable results, the suspension of the test organism was streaked centripetally from the Pyrex cylinder. When plates were seeded, instead of flooded or streaked, with a test organism, it was necessary to carry out the preliminary incubation at refrigerator temperatures and for longer periods. The time of incubation of

the plates after inoculation with the test organism varied in accordance with the growth rate of the organism in question.

Because of the chemical heterogeneity of ustilagic acid (4), the establishment of a more precise quantitative bioassay must necessarily await separation of the components of the mixture, and the determination of the antibiotic activities of each against suitably sensitive microorganisms.

The test organisms most used for streaking have been *Bacillus subtilis* and *Aspergillus niger*, while *Xanthomonas iranslucens cerealis* proved to be an excellent and sensitive organism in seeded plates used for testing paper-strip chromatograms.

For determination of the antifungal and antibacterial spectra, known solutions of ustilagic acid in methanol were added to potato-dextrose agar just before it solidified so that the plates, when poured, contained ustilagic acid in suitable concentrations, e.g. 40, 30, 20, 10, 5, 2.5, 1.25, and 0γ per ml. of agar. At concentrations of about 50γ per ml. and higher, the ustilagic acid crystallized out in the agar, making results obtained by the use of these and higher amounts of the acid in agar of little value.

Toxicity tests were by feeding and intraperitoneal injection of rats and mice.

Experimental

In one series of plates, aliquots of methanolic extract of centrifuged culture mixture were placed in Pyrex cylinders and the plates were then incubated. At the end of one day the first group of plates was streaked with *B. subtilis* and further incubated for 18 hr. when the zones were measured. The second group was similarly treated after two days, the third after three days, etc. It was found that the inhibition zones were increasingly larger in Groups 1 to 4 (days), but were constant in size in Groups 4 to 8 (days). Where the concentrations of ustilagic acid in methanol placed in the cylinders on agar plates ranged from 5.9 mgm. ustilagic acid per cylinder to 82γ per cylinder and the plates were incubated for 24 hr. before streaking with *B. subtilis*, the inhibition zones obtained were approximately equal to each other, and ustilagic acid crystallized in the agar below the cylinder. At 41γ per cylinder, zones were smaller and no ustilagic acid crystallized out. At concentrations of 20γ per cylinder or less, no readable zones were obtained. Concentrations of from 200γ to 50γ per cylinder were useful for assay purposes.

All samples of ustilagic acid produced have shown antibiotic activity against our standard test organisms, *B. subtilis* and *A. niger*. Attempts to separate the activity of the samples from ustilagic acid were unsuccessful. Ustilagic acid was chromatographed on paper with several solvent systems (Table I). Duplicate chromatograms were made in each case; one was employed to determine the position of the ustilagic acid (by painting the paper with bromocresol green) and the other used to determine the position of the antibiotic by placing the paper strip on an agar plate which, after an incubation period, was then streaked on either side of the strip with *B. subtilis* and *A. niger*. In no case was it possible to separate an active principle from ustilagic acid.

TABLE I

PAPER CHROMATOGRAPHY OF USTILAGIC ACID

Solvent	R_F of acid*	R_F of antibiotic
Petroleum ether – ether water	0	0
n-Amyl alcohol – pyridine – water	0.87	0.86
n-Amyl alcohol – 2*N* formic acid	0.80	0.81
n-Amyl alcohol – 0.1 *N* formic acid	0.83	0.80
n-Butyl alcohol – ethanol – water	0.92	0.93

* *R_F = distance travelled up paper strip by solute/distance travelled up the same strip by solvent.*

Recrystallization of ustilagic acid preparations did not alter their antibiotic properties, as tested against *B. subtilis* and *A. niger*. Several samples of the acid were recrystallized from three different methanol–water mixtures and the antibiotic activities of the resultant preparations were compared with those of the original acid samples. Data for one sample are given in Table II in which is listed the relative inhibition zones obtained against *B. subtilis* by the cylinder plate assay method, when the original and the recrystallized samples were assayed. The only change in potency was a small increase in that of the acid recrystallized from 50% methanol, the increase being due, probably, to slight purification of the acid.

TABLE II

ANTIBIOTIC ACTIVITY IN CYLINDER-PLATES OF USTILAGIC ACID RECRYSTALLIZED FROM AQUEOUS METHANOL

Recrystallization solvent, % methanol	Ustilagic acid concentration		
	200 γ/ml.	100γ/ml.	50 γ/ml.
	Inhibition zones against *B. subtilis*, mm.*		
50	40.0	36.0	28.5
30	38.5	35.3	25.8
10	37.3	32.1	25.8
Original ustilagic acid	38.5	32.4	25.8

* *Plates incubated 24 hr. at 30° C. prior to streaking with B. subtilis. Zones read 12 hr. after streaking; 0.5 ml. acid in methanol solution was placed in each cylinder.*

It is of interest to note here that in the precipitation of ustilagic acid from methanol solution by the addition of warm water, the normally long needlelike crystals (Fig. 1) are not always produced. Sometimes the material comes down as long hairlike material and not infrequently as flat, spirally twisted

bands with pointed ends (Fig. 2). These latter forms exhibited antibiotic and physical properties similar to the average material.

Heating aliquots of culture broth for periods of time from 15 min. to one hour at temperatures of 70, 80, 90, and 100° C. did not alter the antibiotic properties of the broth as tested against *B. subtilis* (Table III). Ustilagic acid extracted from broth so treated and from autoclaved broth (15 min. at 15 lb.) showed antibiotic activity against *B. subtilis* and *A. niger* similar to that of material extracted at room temperature or at 50° C. from untreated broth. The heat treatments imposed changed neither the pH (4.6) of the culture mixture used, nor the yields of weighed extracted ustilagic acid. Those yields were in the same order of magnitude in each treatment. That the acid extracted from heat treated samples was essentially unchanged was confirmed by determination of the neutralization and saponification equivalents of each of the samples. Those values were of expected magnitude and showed no pattern of variation.

TABLE III

THE EFFECT OF HEAT TREATMENT OF CULTURE MIXTURE ON THE ANTIBIOTIC ACTIVITY OF THE MIXTURE AND OF THE EXTRACTED USTILAGIC ACID; AND ON YIELDS OF EXTRACTED ACID

Heat treatment of culture mixture (pH 4.6)	Antibiotic activity (mm. zones)			Yields of extracted acid (mgm./ml.)
	Culture mixture (antibact.)	Ustilagic acid		
		Antibacterial	Antifungal	
None*	26.0	29.0	34.0	13.3
30 min. at 50° C.	30.0	30.0	35.5	12.9
60 " " 50° C.	31.0	28.5	33.5	12.4
15 " " 70° C.	29.0	28.5	35.0	13.0
30 " " 70° C.	29.0	29.0	33.5	12.3
60 " " 70° C.	28.5	30.5	34.5	12.9
15 " " 90° C.	32.0	31.5	35.5	12.5
30 " " 90° C.	31.0	30.5	37.5	12.8
60 " " 90° C.	28.0	30.5	36.0	12.0
5 " " 100° C.	29.5	30.5	35.0	13.5
10 " " 100° C.	29.0	29.5	36.5	13.6
20 " " 100° C.	30.0	32.0	37.0	13.2
Autoclaved 15 min. at 15 lb	—	29.3	36.5	12.9

* *Cells killed with toluol.*

The antibiotic spectra have been examined and some of the fungi tested to date are shown in Table IV and some of the bacteria in Table V. The *in vitro* antifungal spectrum appears to be fairly broad, though the antibiotic was not effective against most of the filamentous phycomycetes and penicillia that were tested. In tests of ustilagic acid against certain pathogenic fungi (carried out by Mrs. Julia T. Weld) it was found that ustilagic acid in Sabouraud's agar was approximately twice as potent at room temperature (22°C.) as at 37° C. for *Cryptococcus neoformans* and *Candida albicans*, while

PLATE I

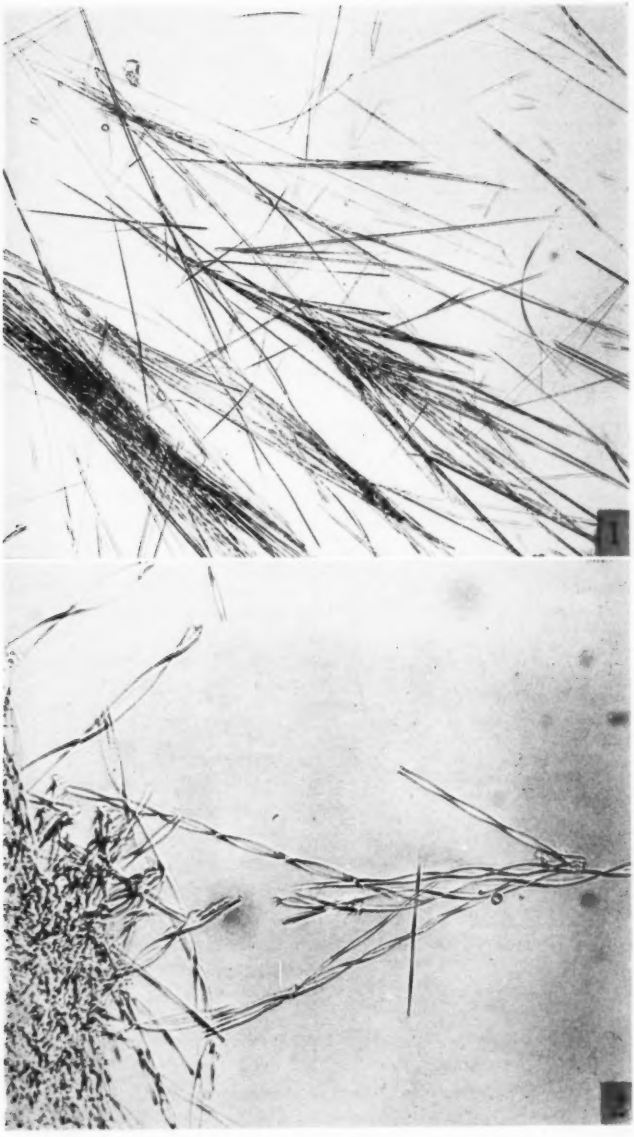

FIG. 1. Normally long needlelike crystals of ustilagic acid precipitated from methanol extract of culture mixture by the addition of warm water.

FIG. 2. Spirally twisted "crystals" of ustilagic acid not infrequently precipitated from methanolic extract of culture mixture by the addition of warm water.

TABLE IV

KNOWN ANTIFUNGAL SPECTRUM OF USTILAGIC ACID, WHEN INCORPORATED IN A POTATO DEXTROSE AGAR ON WHICH TEST ORGANISMS WERE GROWN

P.R.L. No.	Organism	Complete inhibition between (γ per ml. agar)
376	*Streptomyces* sp.	3.1 and 6.3
537	*Thielaviopsis basicola*	3.1 and 6.3
	Streptomyces griseus	5 and 10
—	*Ascochyta* sp.	6.3 and 12.5
530	*Claviceps purpurea*	6.3 and 12.5
482	*Neurospora silophila*	6.3 and 12.5
319	*Chaetomium* sp.	12.5 and 25
534	*Pleospora* sp.	12.5 and 25
533	*Rhizoctonia solani*	12.5 and 25
—	*Aspergillus niger* (spores)	12.5 and 25
—	*A. niger* (mycelium)	25 and 50
417	*Helminthosporium sativum*	25 and 50
26	*Mortierella* sp.	25 and 50
—	*Penicillium* sp.	25 and 50
266	*Stachybotrys* sp.	25 and 50
—	*Verticillium* sp.	25 and 50
369	*Alternaria tenuis*	50—no effect
568	*Armillaria mellea*	50—no effect
86	*Gliocladium roseum*	50—no effect
566	*Polyporus circinatus*	50—no effect
Human pathogens*		
—	*Cryptococcus neoformans*	10 and 15 (Weld)
—	*Candida albicans*	15 and 20 (Weld)
—	*Microsporum Audouini*	(40—slight inhibition —Weld)
—	*M. canis*	(40—no effect—Weld)
—	*Trichophyton rubrum*	(40—slight inhibition —Weld)
—	*T. mentagrophytes*	(40—no effect—Weld
569	*T. mentagrophytes*	(50—no effect)

* *Saubouraud's agar used by Weld.*

other antibiotics tested showed more effect at 37° C. than at 22° C. This may indicate possible usefulness of ustilagic acid in the treatment of moniliasis infections, such as on fingernails, since *C. albicans* here grows at relatively low temperatures. The *in vitro* antibacterial spectrum of ustilagic acid is limited mainly to the Gram-positive bacteria.

Toxicity of Ustilagic Acid

Preliminary toxicity tests show that ustilagic acid is, at least, relatively nontoxic to rats and to mice. Two rats were unaffected by ustilagic acid as 5% of their diet for three weeks, while no ill effects were observed in mice fed ustilagic acid as 1% of their diet for two weeks or injected intraperitoneally with up to 1.5 gm. ustilagic acid per kgm. body weight. The effect of ustilagic acid and of *U. zeae* culture solids on animals will be the subject of a later paper in this series.

TABLE V

KNOWN ANTIBACTERIAL SPECTRUM OF USTILAGIC ACID WHEN INCORPORATED IN A POTATO DEXTROSE AGAR ON WHICH TEST ORGANISMS WERE GROWN

Organism	Gram stain	Complete inhibition between (γ per ml. agar)
Micrococcus pyogenes var. *aureus*	+	5 and 10
Xanthomonas campestris	−	5 and 10
X. translucens cerealis	−	5 and 10
Bacillus subtilis	+	10 and 20
Corynebacterium flaccumfaciens	+	10 and 20
Brucella bronchiseptica	−	20 and 30
Mycobacterium butyricum	+	40—slight effect
Escherichia coli	−	50—no effect
Pseudomonas aeruginosa	−	50—no effect
Aerobacter aerogenes	−	50—no effect

Acknowledgments

The authors are indebted to Mrs. Julia T. Weld, Department of Pathology, College of Physicians and Surgeons, Columbia University, New York, for testing the inhibition *in vitro* of certain pathogenic fungi by ustilagic acid; to the Collip Medical Research Laboratory, University of Western Ontario, London, Ont., for conducting the experiments with rats; and to Dr. J. M. Bell Department of Animal Husbandry, University of Saskatchewan, Saskatoon, Sask., for conducting the tests with mice.

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THE SPLASH-CUPS OF *POLYPORUS CONCHIFER*[1]

By Harold J. Brodie[2]

Abstract

The cup-shaped structures produced by *Polyporus conchifer* are splash-cups from which oidia are thrown to a distance of four feet by raindrops. The oidia are developed on the inner surfaces of the cups in gelatinous masses. The jelly swells at once on being wetted and the cup is filled with oidia which germinate readily and give rise to dikaryotic mycelium. The cups are developed in late summer. From one rim of each cup the flat pore-bearing pileus grows out as the season advances and basidiospores are discharged from the pore layer. During winter, the flat pileus becomes detached from the cup and the latter persists until spring or longer and occasionally may regenerate a new pileus.

The dispersal by rain of oidia from the cups of *P. conchifer* prolongs the reproductive period of the fungus.

Introduction

The vase-shaped fruit bodies of the Nidulariaceae or bird's-nest fungi are now known to be splash-cups from which peridioles are ejected by the action of falling raindrops (1, 3). Likewise, the minute cups containing sclerotia of the fungus *Aleurodiscus Minnsiae* Jackson, belonging to the family Thelephoraceae, are described by Jackson (4) as splash-cups. Thus splash dissemination has been known heretofore to apply to only two families of fungi although it has been recognized by Buller (3) and by the writer (1) among the liverworts, mosses, lichens, and seed plants.

The present paper draws attention to previously unrecognized splash-cups in a species of *Polyporus*.* A third family of fungi, the Polyporaceae, may therefore be added to those in which spore dissemination by means of raindrops is known to occur.

Form and Development of the Fruit Body

Polyporus conchifer (Schw.) Sacc. is a small polypore which grows fairly commonly on dead branches of elm in the United States and Canada. The white or pale buff pilei are thin, semicircular in outline, and about 4 to 5 cm. wide (Fig. 1). On the upper side of the pileus at the base is a small cup-shaped structure. This is at first part of the pileus, the base of the cup being the attachment of the pileus to the branch upon which it grows. The flat expanded portion of the fruit body bears pores on its under surface from which basidiospores are discharged.

In the late autumn, the shelf portion breaks away from the cup (Fig. 3) which persists until the following spring or longer. As a consequence of this abscission, a collection of the fungus made during early autumn usually

[1] *Manuscript received September 28, 1951.*

Contribution from the Department of Botany, Indiana University, Bloomington, Indiana, U.S.A.

[2] *Associate Professor of Botany, Indiana University.*

* *This discovery is also announced in a brief note in Science (2).*

consists of unbroken shelflike fruit bodies (Fig. 1), whereas a collection made in late winter or spring is made up almost wholly of cups (Fig. 2) which bear a strong resemblance to the cups of the common bird's-nest fungus *Crucibulum vulgare* Tul.

The significance of the cups of *P. conchifer* has apparently been a mystery to mycologists in the past. As far as the writer has been able to discover, only Lloyd (5) ever commented upon the unusual nature of a polypore which produces cups in addition to the regular shelving pileus.

Lloyd wrote, in part, as follows: "The sterile portions are the most interesting part for, as far as known, no other species produces similar. They are to me somewhat of a puzzle, for I do not know exactly how or why they are produced."

The resemblance of the cup-shaped remnants of the fruit bodies to cups of the Nidulariaceae is so striking as to suggest that a splash mechanism of some kind is involved. Cups examined in early February of 1951 were found to be empty as were also all others collected during spring and early summer. Whatever material they had contained had evidently been dispersed.

Formation and Dispersal of Oidia

By early August, formation of new fruit bodies was observed. The cup usually developed first and the flat pileus grew out from one side of the cup (Fig. 1, extreme left). The cups always grew only on the upper sides of elm branches (Fig. 6). On Aug. 10 a large number of new cups were found in several stages of development and every new cup contained small dark brown granules of various sizes. The latter were mostly split off from the bottom of the inside of the youngest cups (Fig. 4) but nearer the rim of older cups (Fig. 5).

When a drop of water was placed in a fungus cup being observed under a binocular microscope, the dark granular masses were seen to swell rapidly and almost instantly. From the rapidity with which the dark masses became swollen as they absorbed water, it seems probable that they contain some hydrophilic colloid.

FIGS. 1-6. *Polyporus conchifer.*

FIG. 1. Fruit bodies in various stages of development, showing cupulate basal portion and flat pileate portion which develops as a lateral outgrowth. ×3/2.

FIG. 2. Old fruit bodies collected during winter. The flat pileus has weathered away from each fruit body leaving only the cupulate portion. Such old cups are usually empty. ×2.

FIG. 3. Breaking away of pore-bearing pileate portion of fruit body from cupulate base. This process occurs during the winter. ×4/3.

FIG. 4. Young fruit body in cup stage collected in August. Note dark rim of oidial masses inside cup. When oidia are wetted by rain, they form a spore suspension which is splashed out of the cup by raindrops. ×10.

FIG. 5. Cup older than that shown in Fig. 4. Oidial masses have been formed in concentric zones. ×10.

FIG. 6. A group of young fruit bodies all in the cup stage showing development on upper side of branch. ×7/5.

PLATE I

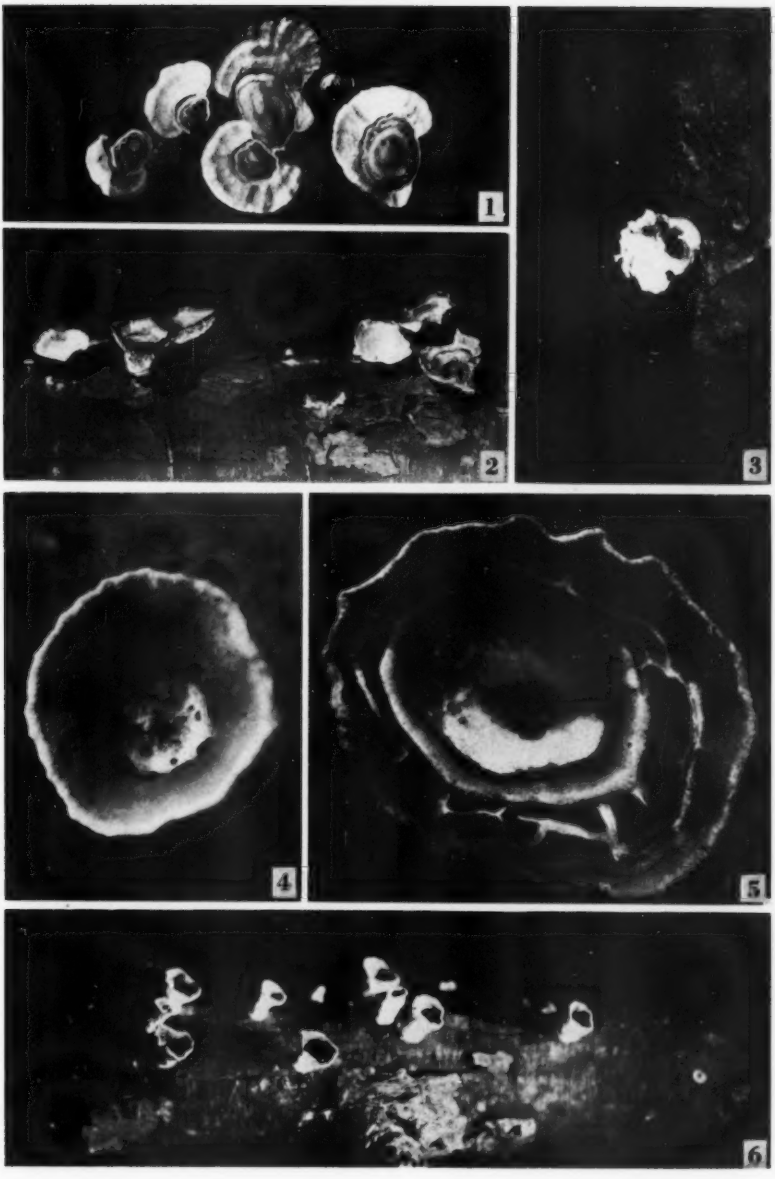
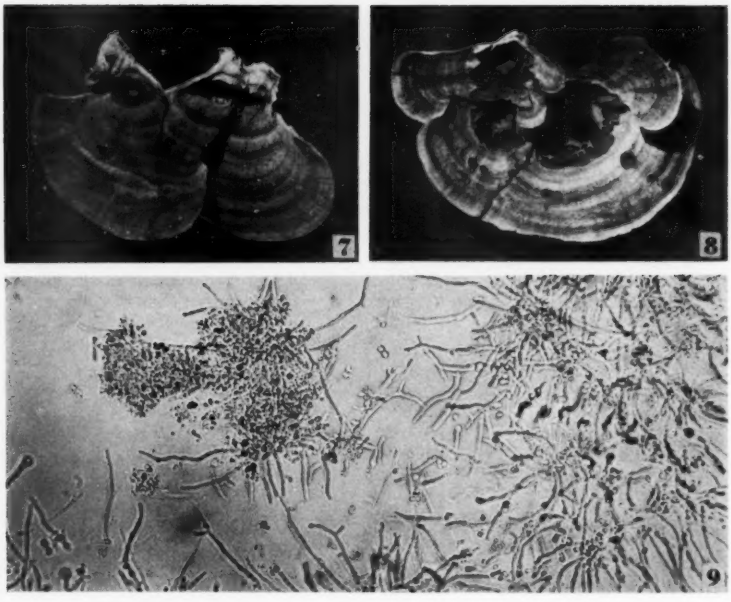


PLATE II



FIGS. 7-9. *Polyporus conchifer.*

FIG. 7. Two confluent fruit bodies showing small splash-cup at base of specimen on left. ×5/3.

FIG. 8. Imbricate fruit bodies showing some very irregular splash-cups at base. Note dark oidial masses at edge of cupulate region. At the right, on the flat pileus, may be seen a single circular splash-cup and two smaller ones are visible at the left. ×1.

FIG. 9. Oidia from splash-cup germinating in hanging drop of nutrient agar. ×250.

The contents of the cup became cloudy as absorption of water progressed and when a drop of the material was examined under the microscope it was found to consist of a suspension of rod-shaped spores 3 μ in diameter and 3 to 8 μ in length.

Transferred at once to a hanging drop of nutrient agar, the spores germinated in 24 hr. at room temperature (Fig. 9) and the germination percentage was very high. Very young germ tubes bore clamp connections which indicated that the spores are dikaryotic.

Most of the spores became separated from one another in water and it was difficult to discover how they had been developed from the inside wall of the cup. In view of their rod-shape and the fact that a few of them remained attached in chains, it may be assumed that they are oidia.

When small drops of water were allowed to fall from a fine tube eight feet into fresh cups in the laboratory, it was observed that oidia were splashed as much as four feet from the cups.

Thus it is clear that the cups of *Polyporus conchifer* are not sterile and functionless as has been assumed but are special organs for the dispersal of oidia by rain. Cup development and the splashing of oidia from the cups occur, for the most part, before the shelf portion of the fruit body has been formed and before basidiospore discharge from the pore layer has begun. In this way, the reproductive period of the fungus is greatly extended. Oidia are splashed from the cups in summer and basidiospores shed from the pilei in the autumn.

Apparently no new oidia are developed in the cups which persist throughout the winter or, if they are, the writer has been unable to find them. A few of the old cups remain alive, however, for one occasionally finds new cups developing from within the old.

In an occasional pileate specimen, one finds shallow circular cups developed on the upper side of the expanded part of the fruit body about half way between the margin and the base (Fig. 8). Masses of oidia were found in the cups on the pileus as well as in the ordinary basal cups. The cups on the pileus are shallow and much more regular in shape than those at the base of the fruit body. They are from 3 to 4 mm. in diameter and perfectly circular in outline. One is reminded by them of the gemma cups of the liverwort *Marchantia*.

General Remarks

It is interesting to note that the splash dispersal mechanism has been developed by fungi in the Polyporaceae as well as in the Nidulariaceae and the Thelephoraceae. The cups of *P. conchifer* are well adapted to splash dispersal; they have the dimensions which appear to be best for the utilization of the force of large raindrops, and the angle at which the sides of the cups are inclined to the vertical is what one finds in such splash-cups as those of *Crucibulum vulgare* (See Brodie (1)).

The cup-producing polypore extends its reproductive period greatly by dispersing oidia by splashing during summer and then discharging basidiospores during autumn when the flat pileus is mature.

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THE EFFECT OF BORON ON THE GROWTH AND SEED PRODUCTION OF ALSIKE CLOVER *TRIFOLIUM HYBRIDUM L.*[1,2]

F. H. MONTGOMERY[3]

Abstract

Alsike clover plants were grown in six different concentrations of boron to study the growth effect of boron on alsike clover and to observe how the intercrossing of plants having different boron content affected seed production. Boron deficiency symptoms occurred in plants grown in nutrient lacking boron, optimum vegetative growth took place in nutrient containing 0.1 to 0.25 p.p.m. boron, and toxicity was observed in plants grown in concentrations of 2.5 and 5.0 p.p.m. boron. Boron was required for flower and seed production, and the optimum level for seed production was 0.5 to 1.0 p.p.m. boron which was higher than that necessary for best vegetative growth. Good numbers of seeds were also obtained when pollen from plants grown in 1.0, 2.5, and 5.0 p.p.m. boron was used on pistils of plants grown in 0.1, 0.25, and 0.5 p.p.m. boron.

Introduction

It is well established that minute quantities of boron are essential for normal vegetative growth of many plants, and much work has been done to determine the optimum amount of this element required for the best development of many economic plants. It has also been shown that good seed production may depend upon the presence of this material in the soil. Brenchley and Warington (3) found that in broad beans only plants receiving sufficient boron formed pods. Grizzard and Matthews (6) report that field plots of alfalfa receiving 15 lb. of borax per acre produced good quantities of seed and hay, while untreated plots produced no seed. Piland, Ireland, and Reisenhauer (10) obtained increased seed production with alfalfa, peanuts, crimson clover, soybeans, and hairy vetch when boron was applied to their experimental soils. Rogers (12) found that the addition of the element to the soil increased seed yield of alfalfa, bur clover, crimson clover, hairy vetch, red clover, and white clover. Davis (4) showed that over a period of four years, boron increased seed production of crimson clover, on an average, from 90 lb. per acre on plots not treated with boron to 850 lb. per acre on plots treated with boron.

Holley and Dulin (9) observed that in cotton a higher boron concentration in the nutrient solution seemed to be required for flower bud development than for growth of vegetative parts. Bobko and Zerling (1) point out that in tobacco, colchicum, and corn there is a greater concentration of boron in the reproductive organs than in the vegetative structures. Considering these observations, it appears possible that increased quantities of boron are

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Contribution from the Dept. of Botany, Ontario Agricultural College, Guelph, Ont.
[2] *A part of the program of the Legume Research Committee in Ontario.*
[3] *Associate Professor of Botany.*

required for proper growth of the stamens and pistils, and for their functioning in the production of seed, than for development of stems and leaves.

The present paper records the results from a greenhouse experiment in which alsike clover plants were grown with six levels of boron supply to obtain data on: (1) the effect of level of supply on vegetative growth and on boron uptake of the plants; (2) the effects on seed production of the boron content of the nutrient solution; and (3) the effects on fertilization of reciprocal cross-pollination of florets from different boron concentrations.

Material and Methods

The experiment was started during the short daylight period of the latter part of December and continued until the middle of July. During the winter months the greenhouse temperatures were kept as near 70° F. as possible. Forty-two pots, 6 in. in diameter, painted inside with black asphaltum, were filled with pure, washed quartz sand, and planted with seed of alsike clover. These were watered daily from the beginning of the experiment with the nutrient solution of Hoagland and Arnon (7), and once a week the pots were thoroughly flushed with distilled water. The chemicals used in the preparation of the nutrient solution were c.p. quality, since preliminary experiments indicated that boron deficiency could be induced by the use of this grade of chemicals. To separate aliquots of the nutrient, boric acid was added so that boron was present in the concentrations of 0, 0.1, 0.25, 0.5, 1.0, 2.5, and 5.0 p.p.m. After the plants were well developed, they were thinned to one per pot, six replications were included for each of the boron concentrations, and the whole series of pots was arranged in a Youden square (15) in the greenhouse.

At the time of pollinations, all except 10 florets on a head were removed, and these 10 florets were hand pollinated. Emasculations of flowers were not made since Riddell (11) showed that alsike clover was highly infertile to self-pollination. The pollinations were made so that some florets of plants growing in each boron concentration received pollen from plants of the same boron concentration, and other florets were crossed with pollen from plants growing in each of the other boron strengths. Thus flowers within each boron level were pollinated between themselves, and with all of the other boron levels. The only exception was those plants receiving no boron, and these did not produce sufficient flowers to carry out the complete series of crosses. Enough flowers for two pollinations, namely 0×0 and 0×0.5 were available. Ten replications were made of each crossing, so that 100 florets were pollinated within each series of crosses. Self-pollinations were also made to verify the necessity for cross-pollination in alsike clover. Five thousand pollinations were made within one week to eliminate as far as possible the variation of fertility that may occur in plants at different periods of flowering.

Green and dry weights of tops were obtained, counts were made of the number of florets per head, and the weights of heads produced by the plants grown in the different boron concentrations were determined. The boron

content of the tops and flowers was determined by a modified quinalizarin method. Counts were made of the seeds produced by the different cross-pollinations and self-pollinations, and all data were subjected to statistical analysis.

Pollen from heads containing the least boron and from heads containing the most boron was germinated on culture medium containing no boron and medium with 50 p.p.m. boron. The number of pollen grains to develop pollen tubes, and the effects of boron on the growth of the pollen tubes were observed.

Results

The alsike seeds germinated equally well in all concentrations of boron used in the nutrient solutions. The seedlings developed normally for about 60 days, at which time leaflets of the older leaves of plants receiving 5.0 p.p.m. boron showed yellowing around the margins, indicating boron toxicity. Later, the plants receiving 2.5 p.p.m. boron showed a similar yellowing. Apart from this characteristic, the plants showing toxicity developed normally and produced abundant flowers. None of the plants in concentrations lower than 2.5 p.p.m. showed toxicity symptoms. Plants grown in nutrient containing 0.1, 0.25, 0.5, and 1.0 p.p.m. boron at no time showed any abnormalities that could be attributed to boron.

When no boron was supplied, the plants were dwarfed, the leaflets acquired interveinal yellowing and often a bronzed appearance, while the veins retained their normal green color. Curling, wrinkling, and eventual dying of the leaflets took place, and rosetting of the leaves occurred at the tips of the branches. Flower peduncles were short, few flower heads developed, and, of those that did form, many turned brown and died before the florets reached maturity.

Chemical analysis at late flowering stage, Table I, showed that both p.p.m. and mgm. boron in the tops and heads increased with the increase of boron in the nutrient solution. The only exception was in mgm. boron in heads of

TABLE I

BORON CONTENT OF THE TOPS AND HEADS OF ALSIKE CLOVER AT THE LATE FLOWERING STAGE AND GROWN IN DIFFERENT BORON CONCENTRATIONS

Boron in nutrient soln. (p.p.m.)	0	0.1	0.25	0.5	1.0	2.5	5.0	r
Tops								
Boron in mgm.	1.10	2.99	4.73	6.27	7.44	11.94	18.48	0.978**
Boron in p.p.m.	16.2	22.7	37.0	49.8	68.9	106.8	174.3	0.989**
Heads								
Boron in mgm.		0.022	0.024	0.026	0.028	0.039	0.034	0.785
Boron in p.p.m.		36.5	46.9	59.4	61.5	63.7	75.9	0.836*

* *Significant at 5% level.*
** *Significant at 1% level.*

plants grown in the 5.0 p.p.m. boron nutrient. The high correlations confirm the proportional increase of boron in the tops and heads with the increase of boron in the nutrient.

Table II and Text-fig. 1 present the average green and dry weights of plants grown in the different boron levels. The greatest weight difference was between plants grown in 0 and 0.1 p.p.m. boron, and it is evident that boron

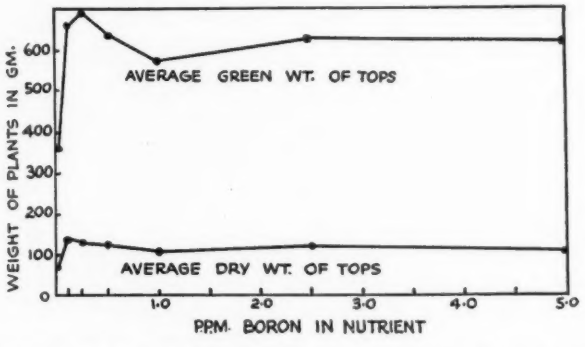
TABLE II

AVERAGE GREEN AND DRY WEIGHTS OF ALSIKE CLOVER PLANTS GROWN IN DIFFERENT BORON CONCENTRATIONS

Boron in nutrient soln. (p.p.m.)	0	0.1	0.25	0.5	1.0	2.5	5.0	r
Av. green wt. in gm.	355.7	663.6	696.5	643.1	573.5	620.8	610.1	0.143
Av. dry wt. in gm.	68.1	131.9	127.8	126.0	108.0	111.8	106.0	0.065

NOTE: *Necessary mean diff., green wt., 5% level, 100.93; 1% 136.37.*
Necessary mean diff., dry wt., 5% level, 20.35; 1%, 27.54.

was essential for good vegetative growth. Failure of the differences in weights above the 0.1 p.p.m. level to very definitely exceed the necessary mean differences shows that additional boron above this level did not change



TEXT-FIG 1. Average green and dry weights of tops of alsike clover plants grown in different boron concentrations.

significantly the vegetative growth. No proportional variation between the weights and the changes of boron in the nutrient was observed, and this was also seen from the low correlation coefficients obtained.

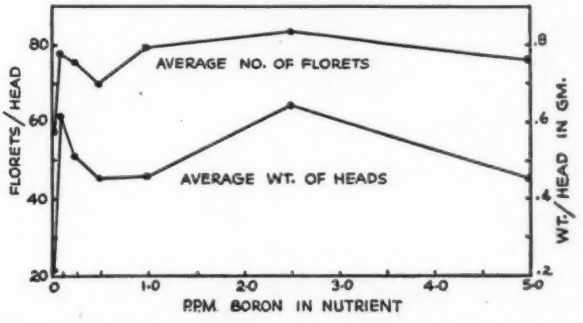
A similar trend in results appears in Table III and Text-fig 2 for the average number of florets per head and the average weights of heads of the plants.

TABLE III

AVERAGE NUMBER OF FLORETS PER HEAD AND AVERAGE WEIGHT PER HEAD FROM PLANTS GROWN AT DIFFERENT BORON LEVELS

Boron in nutrient soln. (p.p.m.)	0	0.1	0.25	0.5	1.0	2.5	5.0	r
Av. no. of florets per head	57.3	78.3	76.1	69.9	79.3	82.0	76.4	0.376
Av. wt. of heads in gm.	0.21	0.61	0.51	0.45	0.46	0.62	0.45	0.180

NOTE: *Necessary mean diff. for florets, 5%, 9.70; 1% 12.94.*
Necessary mean diff. for wt. of heads, 5%, 0.10; 1% 0.14.



TEXT-FIG 2. Average number of florets per head and average weights of heads of alsike clover plants grown in different boron concentrations.

The seed production by the different cross-pollinations and statistical data are given in Table IV. Text-fig 3 shows the regression of seed production on the boron in the nutrient solution for the plants used as source of pistils, and Text-fig 4 shows this regression for the plants used as source of pollen. Crossing of florets grown in the zero boron nutrient produced the least seed, 120 seeds per 100 florets, but when pistils from the same nutrient were pollinated with pollen from the 0.5 p.p.m. boron plants, the number of seeds increased to 247 seeds per 100 florets. This was higher than the mean production of 234.9 seeds per 100 florets for the whole experiment.

The totals of the 1.0 p.p.m. column and the 0.5 p.p.m. row of Table IV indicate that the optimum amount of boron in the nutrient for seed production was at the 0.5 to 1.0 p.p.m. boron levels. A concentration of high values is found in the block of figures enclosed by lines, that is, pollen from the plants of the 1.0, 2.5, and 5.0 p.p.m. boron nutrient, on pistils from the 0.1, 0.25, and 0.5 p.p.m. boron nutrient gave good seed production. The correlation coefficients between boron in the nutrient and the seed production were low, and the numbers of seeds produced did not vary proportionally with the changes in the boron content of the nutrient.

TABLE IV

SEEDS PRODUCED PER 10 HEADS OF 10 FLORETS EACH BY ALSIKE CLOVER BY CROSS-POLLINATION OF FLOWERS GROWN IN VARIOUS BORON CONCENTRATIONS

Boron in nutrient soln. (p.p.m.)	Male plants								
	0	0.1	0.25	0.5	1.0	2.5	5.0	Total seeds	Means
Female plants									
0	120†			247†					
0.1		217	257	214	285	262	283	1518	253.00
0.25		211	204	217	283	264	232	1411	235.16
0.5		232	227	259	263	281	267	1529	254.83
1.0		230	207	241	253	210	178	1319	219.83
2.5		198	179	194	184	220	204	1179	196.50
5.0		216	292	233	264	255	243	1503	250.50
Total seeds		1304	1366	1358	1532	1492	1407		
Means		217.33	227.66	226.33	255.33	248.66	234.50		234.97

Analysis of variance

Sources of var.	Deg. F.	Sums of sq.	Mean sq.
Male treatment	5	624.88	124.97**
Female treatment	5	1601.95	320.39**
Interaction	25	1400.67	56.02*
Error	324	10,804	33.34

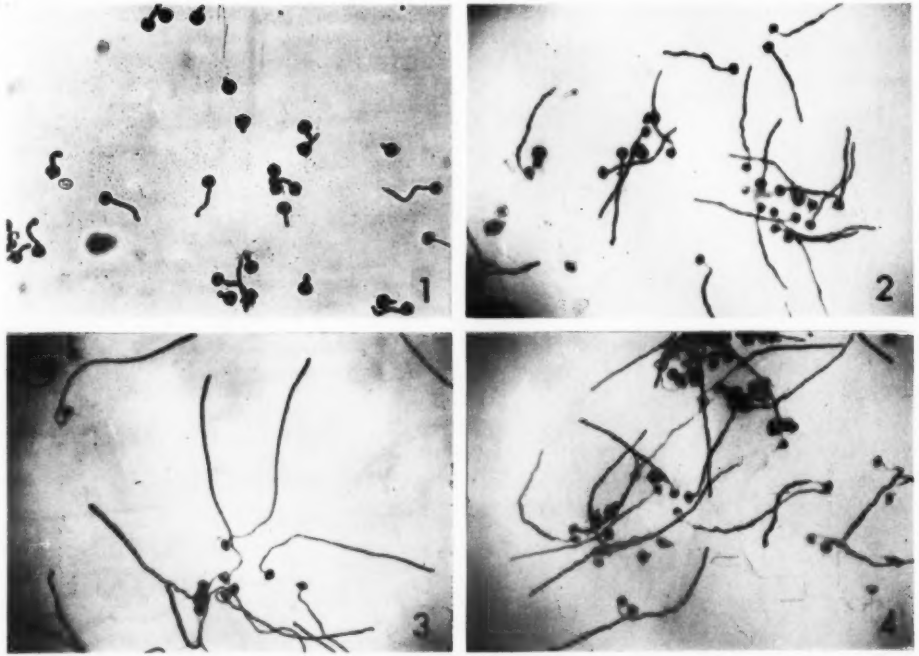
† *Not included in analysis of variance.*

NOTE: *Necessary mean diff. 5% level = 6.52.*
Necessary mean diff. 1% level = 8.62.
r for seeds and boron in nutrient of pollen = 0.323.
r for seeds and boron in nutrient of pistils = −0.099.

When the above relationships between boron nutrition of male and female parents and seed production were observed, possible factors that might cause these relationships were investigated. Since Bobko and Zerling (1), Schmucker (13), and others showed that boron influenced the germination of pollen, pollen from plants grown in zero boron and 5.0 p.p.m. boron was cultured on medium containing no boron and 50 p.p.m. boron. Five hundred pollen grains were examined from each test and the numbers of these to germinate and the character of the pollen tubes were observed.

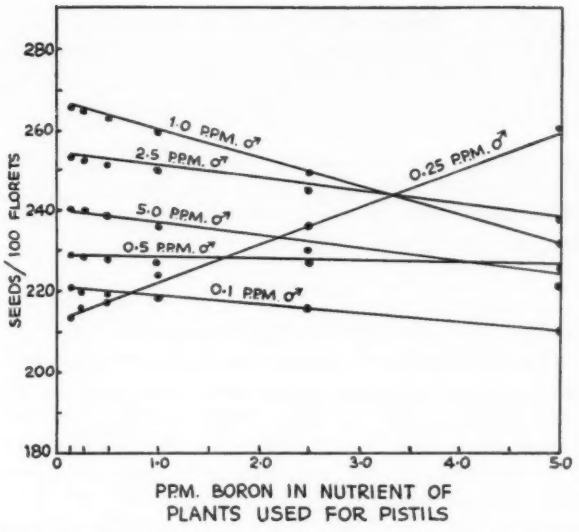
When pollen from the zero boron level was placed on nutrient with no boron, poor germination occurred and the pollen tubes were short and poorly formed, Table V and Plate I, Fig. 1. Pollen from the same heads germinated well on medium containing 50 p.p.m. boron and had longer tubes but distortion of the tubes was still evident, Plate I, Fig. 2. Pollen from plants of high boron nutrient gave good germination and normal pollen tubes on medium with no

PLATE I



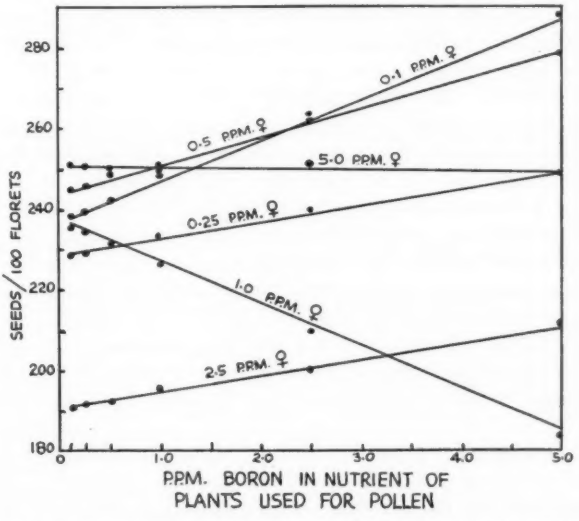
Germination of pollen from 0 and 5.0 p.p.m. boron nutrient plants on 0 and 50 p.p.m. boron medium. (× 80)

FIG. 1. Pollen from 0 boron nutrient plants on 0 boron medium. FIG. 2. Pollen from 0 boron nutrient plants on 50 p.p.m. boron medium. FIG. 3. Pollen from 5.0 p.p.m. boron nutrient plants on 0 boron medium. FIG. 4. Pollen from 5.0 p.p.m. boron nutrient plants on 50 p.p.m. boron medium.



TEXT-FIG. 3. Regression of seed production on p.p.m. boron in the nutrient solution of plants used as source of pistils.

s_b* for 0.1 p.p.m. = 9.1; 0.25 p.p.m. = 7.1; 0.5 p.p.m. = 10.0
1.0 p.p.m. = 3.1; 2.5 p.p.m. = 9.6; 5.0 p.p.m. = 5.9.



TEXT-FIG. 4. Regression of seed production on p.p.m. boron in the nutrient solution of plants used as source of pollen.

s_b* for 0.1 p.p.m. = 6.4; 0.25 p.p.m. = 8.0; 0.5 p.p.m. = 4.2;
1.0 p.p.m. = 4.7; 2.5 p.p.m. = 3.2; 5.0 p.p.m. = 6.9.

* s_b = Standard error of the regression coefficient in per cent.

boron, Plate I, Fig. 3. Similar pollen placed on high boron medium gave numerous tubes but they were abnormal in appearance, Plate I, Fig. 4. A supplementary test of pollen on medium with 100 p.p.m. boron showed that this concentration was toxic and the few tubes that developed were short and abnormal.

Plants that were self-pollinated gave low numbers of seeds and, from the 700 florets thus treated, 49 pods and 100 seeds were obtained. On comparing this with seed developed when cross-pollinations were made, it is evident that cross-pollination was the more advantageous.

TABLE V

GERMINATION OF 500 ALSIKE POLLEN GRAINS ON BORON NUTRIENT

Boron in nutrient of plants (p.p.m.)	Boron in germinating medium (p.p.m.)	
	0	50
0	191	413
5	333	391

Discussion

Germination of seeds and development of seedlings were not affected by any of the boron concentrations used in the experiment. The delayed appearance of boron deficiency symptoms can be attributed to the daily feeding of nutrient to the plants. Brenchley and Warington (3) found that broad bean plants fed by the drip method had boron deficiency symptoms delayed from three to six weeks in comparison with plants receiving nutrient at intervals of 8 to 16 days. They came to the conclusion that the keeping of a constant balance in the nutrient solutions in the pots promoted growth for a longer period before deficiency symptoms became evident. Brenchley (2) considered that, if plants are growing vigorously, they are able to withstand more boron than when growth is slow. The plants of this experiment grew very rapidly in the greenhouse and this would delay the boron toxicity symptoms.

No conclusion could be reached as to the variation in flowering time within the boron concentrations. Since only six plants were used with each series, it was not possible to say that differences in flowering time were caused by boron alone.

Warington (14) showed for broad beans double peaks of growth when boric acid was used at concentrations of 1 : 12,500,000 and 1 : 50,000. Eaton (5) indicated that there are two levels of boron concentration for the maximum growth of sunflower plants. He did not consider the second peak at the 0.25 p.p.m. level to be caused by boron. Hodgkiss *et al.* (8) showed similar double growth peaks for soybeans, but they considered these to be due to boron effects.

Figs. 1 and 2, showing green and dry weights and the number of florets per head and weights of heads, present similar double maxima. However, in this experiment, the differences above the 0.1 p.p.m. level were not pronounced enough to statistically establish that there are two maximum levels of boron for alsike clover development.

The production of the lowest number of flowers and seeds by the intercrossing of the zero boron series, demonstrates well the necessity of this element for the normal cycle of growth in alsike clover.

The pollen germination experiment would suggest that boron nutrition of pollen was a major factor in the cross-pollinations. Low boron pollen on low boron pistils produced little seed because of the poor germination and poor tubes. Better results were obtained by low boron pollen on high boron pistils, and high boron pollen on high boron pistils because of the greater germination, but abnormal pollen tubes interfered with proper seed set. The formation of well developed, normal pollen tubes by the high boron pollen on low pistils accounted for the block of high values referred to above. This would also indicate that, under field conditions, with variable boron nutrition, a high boron level in the pollen parent would compensate for poor boron supply in the pistil parent growing elsewhere in the field. The optimum condition, however, would be when both pollen and pistils contained boron equivalent to that of plants grown in 0.5 to 1.0 p.p.m. boron, i.e. 50 to 70 p.p.m.

It would seem that the healthy appearance of alsike clover plants is not necessarily indicative of the seed production, since in the experiment the best vegetative growth took place at lower boron levels than the best seed production.

Acknowledgments

I gratefully acknowledge the generous assistance and advice of Dr. R. O. Bibbey in the preparation of this paper.

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MUTATIONS IN POLYPLOID CEREALS

IV. A SEGMENTAL CHROMOSOME DEFICIENCY DETERMINING AN α SERIES RATIO IN A SPELTOID WHEAT[1]

By Stanley G. Smith[2]

Abstract

The C chromosome of beardless *Triticum vulgare* carries epistatic inhibitors, about 30 crossover units apart on the longer arm of the chromosome, which prevent the expression of the bearded-speltoid complex of genes. It has earlier been shown that het speltoid mutants may lack one of the two sets of inhibitors through the loss of the entire C chromosome, or its longer arm, or an interstitial segment of this arm. The first determines Series β segregation (1 normal : ca. 5 het speltoids : ca. 0.03 speltoids), the last two Series γ (1 : 1 : ca. 0.05). A third type of progeny ratio, α, is a distortion of a 1 : 2 : 1 caused by a greater or lesser deficiency of speltoids and, less markedly, of het speltoids. Some workers regard α ratios as resulting from mutation of the epistatic inhibitors to their recessive state; others as due to chromosome aberration similar to, but less extensive than, that observed in typical γ strains. The latter interpretation is proved correct herein. That deviations from the ratios characteristic of the three Series are determined by the degree of pollen certation superimposed on the proportion in which normal and mutant ovules are formed has been shown by analyses involving natural hybrids and crosses made with other types. The extent of certation in α and γ Series may be measured from their progeny ratios—normal pollen = N + H − Sp : mutant pollen = 2 × Sp. A rough correlation has been established between the length of the mutant C chromosome, percentage conjugation with C, proportion of N : Sp progeny, percentage β het Sp progeny, and the coefficient of certation. The percentage het Sp of Series β expected as secondary mutants in the het Sp progeny of α and γ het Sp approximates to $\frac{H - (N + Sp)}{H}$.

I. Introduction

The general problems associated with the origin and behavior of speltoid mutations have been outlined by Huskins and Sander (4) and treated in detail by Smith, Huskins, and Sander (11). It has been shown that the speltoid-bearding complex is commonly inherited in a unitary manner: in Series β as the result of the loss of a whole C chromosome (chromosome IX in the terminology of Sears (7)); in Series γ either by the loss of an interstitial portion of the major arm of a C chromosome or by the complete loss of this arm; and, finally, in Series α most probably by the loss of a region too small to be demonstrated at the first metaphase of pollen-mother-cell meiosis.

By crossing part mutations (bearded normal and beardless speltoid) Nilsson-Ehle (6) has shown that the two factors are between 27 and 36 cross over units apart—a conclusion substantiated by Watkins (14) in finding 28–39% crossing over in intervarietal and interspecific crosses. The occurrence of crossing over in beardless heterozygous speltoids synthesized in this manner and its absence from beardless heterozygous speltoids that have arisen in a single step is strong a priori evidence that the latter result from a single

[1] *Manuscript received July 25, 1951.*

[2] *Cytogeneticist, Forest Insect Laboratory, Sault Ste. Marie, Ont. Formerly of the Department of Genetics, McGill University, Montreal, P.Q.*

relatively extensive deficiency rather than from simultaneous point mutations. In Watkin's (14) terminology heterozygous speltoids of both Series α and Series γ can, as has already been shown (11), be symbolized as

$$\frac{\text{B } 27+ \text{ k}}{- - - - - - -},$$

where B and k represent factors on the C chromosome epistatic to the bearding and speltoid glume factors situated on one or more other pairs of chromosomes. On this basis and unlike β heterozygous speltoids which lack one C chromosome,

$$\frac{\text{B } 27+ \text{ k}}{- - - - - - - - -},$$

the difference between α and γ heterozygous speltoids is one mainly of degree, and in segregation all manner of ratios intermediate between 1 : 2 : 1 and 1 : 1 : 0 are theoretically possible. Whether a 42-chromosome heterozygous speltoid gives α or γ segregation is dependent primarily upon the frequency with which mutant male gametes function in competition with normal ones (certation). It might be expected, then, that pollen with a large deficiency from the C chromosome would suffer from certation more than that with a smaller deficiency and thus provide an inverse correlation between the extent of visible deficiency and the frequency with which speltoids are produced. Limited evidence that such is the case will be provided later.

The present problem is concerned with the cytogenetics of a strain (α3 herein) which genetically deviates somewhat from other established α strains. In addition, it gave rise to a beardless* speltoid substrain which, though in general breeding true to beardlessness, on occasions produced bearded speltoids in later generations. A second problem therefore concerns the origin and relative instability of this beardless form.

On the basis of its segregating in conformity with Nilsson-Ehle's (5) definition, Huskins (2) classified the parental strain as Series A (now termed Series α). His classification was founded also on the observation of certain multivalent associations which, however, he has since (3) concluded are independent of Series α segregation. The assignment to Series α is strongly supported by the increased vigor and fertility of the speltoid segregates relative to those of other strains studied by Huskins which quite obviously belonged to Series γ. There has, however, in all generations, been a consistent shortage of speltoid segregates and a corresponding excess of normals of a degree which has distinguished this strain from others of Series α.

In 1929, heads of first generation heterozygous speltoids were supplied to Dr. Isamu Uchikawa, Kyoto, Japan. Uchikawa's first generation segregation ratio from them is not given in his paper (13), but the grouped total for the four succeeding years is not significantly different from a 1 : 2 : 1 ratio ($\chi^2 = 6.515$; $P = <.05$) although there is the usual deficiency of speltoids characteristic

* *This term is used in a colloquial sense—See (4).*

of α strains. As will be shown later there are grounds for believing that the plants he selected from his first generation for further breeding were genetically distinct from the material studied herein.

II. Material and Methods

The material, originating from Dr. Å. Åkerman's heterozygous speltoid strain 1924–443, was obtained by Dr. C. L. Huskins in 1926. It consisted of one normal (26–57), one bearded speltoid (26–56), and four heterozygous speltoids (26–58/61). At that time Dr. Åkerman stated that this strain of spring wheat had been a constant ratio-type for many years and came originally from the cross Brown Schlanstedt × "0715"; no other information was supplied.

In a recent publication, Åkerman and Mac Key (15) have given the detailed ancestry and sib-progeny records of the material discussed here. From some 12,000 plants they obtained a ratio of 1 : 1.24 : 0.26 and consider it a very constant A type ". . . . with a relatively low frequency of het speltoids". They attribute the deviation from a 1 : 2 : 1 progeny ratio to certation.

The cultivation and cytological methods used herein are precisely the same as those described in earlier papers of this series (11, 12), as are the majority of the abbreviations—others are explained in the text. The genetical data have been provided by Dr. C. L. Huskins who made the necessary crosses and grew the plants of the first two generations at the John Innes Institution, Merton Park, England, in 1929 and 1930.

All drawings were made using a 1.5 mm., 1.3 N.A. Zeiss apochromatic objective and a 10× ocular for complete nuclei or a 20× for individual configurations. These give bench-level magnifications of 2200 and 4400 diameters; in reproduction the illustrations have been reduced to ×1100 and ×2200.

III. Observations

To facilitate description the material will be considered as consisting of the original bearded (bb) speltoid-producing strain $\alpha 3$, and two substrains: (1) the progeny of a het speltoid descendant of a bearded speltoid, and (2) the progeny of a beardless speltoid sib of this het speltoid.

(i) The Bearded Strain

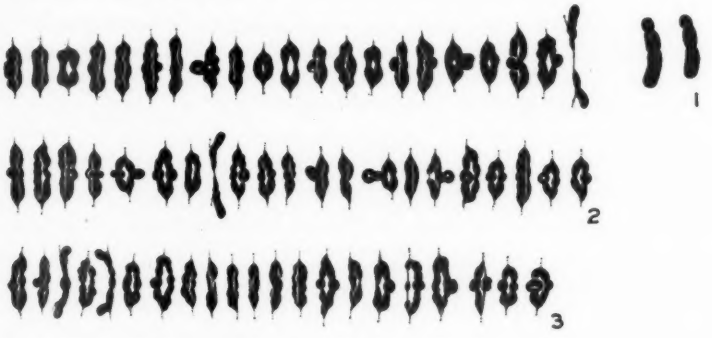
(*a*) *Genetical*

The original het speltoids and their het speltoid progeny gave a total of 237 N, 317 H, and 68 bb Sp (Table I, 6) or 10.9% bearded speltoids. Partly as a result of the increased frequency of the het speltoids, but mainly because of the high proportion of speltoids, there is a clear approach to Series α. That the deviation from a 1 : 2 : 1 ratio ($\chi^2 = 92.067$) is not the result of differential mortality is shown by the fact that even if the 99 seeds which failed to give mature plants were all speltoids the deviation would still be statistically significant ($\chi^2 = 24.090$).

The normals bred true, but in the D_2 generation one of the bearded speltoids produced four het speltoids in addition to 15 bearded speltoids. In view of the appearance of the former and in conformity with other cases (11), these are considered to have resulted from natural crossing with other plants of the same strain. Again in the D_2, another bearded speltoid produced one beardless speltoid and, in a resowing, one het speltoid along with a total of 101 bearded speltoids. The progeny of the het speltoid constitutes Substrain 1; that of the beardless speltoid Substrain 2. The bearded speltoids are quite vigorous and produce mostly viable seeds (Table I, 13–15) in keeping with the α classification.

(*b*) *Cytological*

Apart from the speltoids which gave the two substrains, the only family propagated after 1928 was derived from one of their het speltoid sibs. The constitution of this het speltoid was determined from acetocarmine smears prepared from dissolved paraffin-embedded material (for technique, See (11)). It had 21 bivalents at the first metaphase, as did two of its het speltoid progeny. However, they were not all homomorphic but included one heteromorphic pair, conjugated in 70 nuclei (Fig. 1*a*) and unpaired in five (Fig. 1*b*; Photomicrograph Fig. 13). Especially in the latter case it is clear that one of these is a normal C chromosome and that the other, in being shorter between the secondary constriction and the distal end, resembles the Cd chromosome characteristic of γ speltoids (11).



FIGS. 1 to 3. Segregates of parent strain α3:

1 *a*. Het speltoid with 20_{II} + CCd heteromorphic bivalent.
1 *b*. Het speltoid with the C and Cd unpaired.
2. Normal with 21_{II}.
3. Bearded speltoid with 21_{II}.

Three normal segregates of the parent plant had 42 chromosomes. These formed 21 homomorphic bivalents (Fig. 2) in 28 of the 30 nuclei examined. In the remaining two an association of four chromosomes occurred (Figs. 4*a* and *b*).

TABLE I

PROGENY RECORD OF PARENT STRAIN, SUBSTRAIN 1, AND SUBSTRAIN 2

Parent	Year grown	Gener. No.	Sown	No. of fams.		No. seeds		N	H	B'dd Sp	B'less Sp	Total
						Sown	Germ.					
1. Original H 26–58/61	1926	D_1	1927	4	—	166	148	54	74	18	—	146
2. H segregates	1927	D_2	1928	14	—	480	441	168	228	42	—	438
3. Resowing			1930	—	1	3	3	3	0	0	—	3
4. Resowing			1935	—	1	36	22	7	7	3	—	17
5. Resowing			1937	—	1	36	18	5	8	5	—	18
6. Total				18	3	721	632	237	317	68	—	622
7. Original N 26–57	1926	D_1	1927	1	—	20	20	20	—	—	—	20
8. N ex 26–58/60	1927	D_2	1928	5	—	60	54	54	—	—	—	54
9. N head of chimera				1	—	36	29	29	—	—	—	29
10. H head of chimera				1	—	24	22	22	—	—	—	22
11. N ex H	1930	D_3	1936	1	—	24	8	8	—	—	—	8
12. Total				9	—	164	133	133	—	—	—	133
13. Original Sp 26–56	1926	D_1	1927	1	—	20	20	—	—	20	—	20
14. Sp ex 26–58/60	1927	D_2	1928	3	—	71	65	—	4	60	1	65
15. Resowing			1930	—	1	85	81	—	1	80	—	81
16. Sp ex Sp	1930	D_3	1936	1	—	12	3	—	—	3	—	3
17. Total				5	1	188	169	—	5	163	1	169
Substrain 1												
18. H ex B'dd Sp	1930	D_2	1931	1	—	72	69	28	37	3	—	68
19. H ex H nat. cross	1931	D_4	1932	3	—	84	70	30	15	11	—	56
20. H segregates	1932	D_5	1936	4	—	144	47	18	26	2	—	46
21. Total				8	—	300	186	76	78	16	—	170

TABLE I—*Continued*

PROGENY RECORD OF PARENT STRAIN, SUBSTRAIN 1, AND SUBSTRAIN 2—*Continued*

Parent	Year grown	Gener. No.	Sown	No. of fams.	No. seeds		N	H	B'dd Sp	B'less Sp	Total
					Sown	Germ.					
Substrain 1—*Concluded*											
22. N ex H ex B'dd Sp	1931	D_4	1932	2 —	48	38	32	1	—	—	33
23. N ex N	1932	D_5	1936	2 —	48	15	12	1	—	—	13
24. Total				4 —	96	53	44	2	—	—	46
25. B'dd Sp ex H ex B'dd	1931	D_4	1932	2 —	48	34	—	—	25	—	25
26. B'dd Sp ex B'dd ex H	1932	D_5	1936	2 —	24	4	—	—	3	—	3
27. Total				4 —	72	38	—	—	28	—	28
28. H ex N ex H	1932	D_5	1936	1 —	36	22	11	9	2	—	22
29. H ex N ex N	1936	D_6	1940	1 —	48	42	14	20	5	—	39
30. Total				2 —	84	64	25	29	7	—	61
Substrain 2											
31. B'less Sp ex B'dd	1928	D_3	1929	1 —	36	33	—	—	—	33	33
32. Resowing			1930	— 1	49	47	—	—	5	42	47
33. B'less Sp ex B'less	1929	D_4	1930	1 —	24	24	—	—	1	23	24
34.	1930		1936	1 —	36	18	—	—	2	13	15
35.	1929		1930	1 —	24	24	—	—	—	24	24
36.	1930		1931	1 —	23	23	—	—	—	23	23
37.	1931	D_5	1932	1 —	24	21	—	—	—	16	16
38.	1932	D_6	1936	1 —	12	3	—	—	—	3	3
39. B'dd Sp ex B'less	1930	D_4	1931	6 —	144	134	—	—	131	1	132
40. B'dd Sp ex B'dd	1931	D_5	1932	2 —	48	33	—	—	25	—	25
41.	1932	D_6	1936	1 —	12	4	—	—	4	—	4
42. B'less Sp ex B'dd ex B'less		D_5	1932	1 —	24	24	—	—	5	13	18

TABLE I—*Concluded*

PROGENY RECORD OF PARENT STRAIN, SUBSTRAIN 1, AND SUBSTRAIN 2—*Concluded*

Parent	Year grown	Gener. No.	Sown	No. of fams.	No. seeds		N	H	B'dd Sp	B'less Sp	Total
					Sown	Germ.					
Substrain 2—*Concluded*											
43. Resowing	1931		1936	— 1	36	16	—	—	1	14	15
44. B'less Sp ex B'less	1932	D_6	1936	1 —	32	14	—	—	—	14	14
45.	1936	D_7	1937	1 —	25	15	—	—	4	11	15
46. Total seg. B'less				5 2	230	177	—	—	18	149	167
47. Total nonseg. B'less				5 —	115	85	—	—	—	80	80
48. Total B'dd Sp				9 —	204	171	—	—	160	1	161

A bearded speltoid sib of the beardless speltoid had 21 homomorphic bivalents (Fig. 3). It is concluded, therefore, that the normals, het speltoids, and bearded speltoids differ in possessing 2 C, 1 C + 1 Cd, and 2 Cd chromosomes respectively. The presence at the first metaphase of two Cd chromosomes in speltoids, or for that matter two C chromosomes in normals, is difficult to demonstrate since it is only when they are morphologically different or present as univalents that they can be recognized. This conclusion is confirmed, however, by the occurrence in the progeny of a bearded speltoid of a het speltoid; it had two unequal chromosomes which were clearly C and Cd. This plant presumably arose by natural crossing, a 20 + C male gamete being introduced from a related plant.

(ii) The Bearded Substrain 1

(*a*) *Genetical*

The parent het speltoid that arose from a bearded speltoid and seven of its descendent het speltoids together gave 76 N, 78 H, and 16 bb Sp (Table I, 21). This looks like a γ ratio apart from the high frequency of speltoids. The parental plant was originally classified as a "possible beardless speltoid", but, since its descendants showed evidence of a *T. spelta* ancestry in the shape of the glumes, it is clearly a natural hybrid with an unrelated strain. The value of χ^2 (2.456, Table IV, 1), however, shows that it is not statistically different from the parent strain which was classed as α.

Four normals gave two het speltoids in addition to 44 normals (Table I, 24). The two het speltoids (Table I, 30) in turn gave ratios close to that of this substrain (Table IV, 2), so that they apparently resulted from natural crossing within the substrain.

Four segregated bearded speltoids were propagated. Germination from two was poor, presumably owing to delay in sowing, and from the four only 28 plants matured, all bearded speltoids, from the 72 seeds sown (Table I, 27).

(*b*) *Cytological*

The parent het speltoid and eight of its het speltoid progeny possessed 42 chromosomes, including a heteromorphic pair. In 131 nuclei the heteromorphic chromosomes formed a bivalent (Fig. 5; Photomicrograph Fig. 14); in 12 there were two unequal univalents, morphologically like the C and Cd chromosomes of the parent strain. A similar CCd pair was present in the one examined of the two het speltoid progeny of normals. Two other chromosomes were unpaired in three nuclei and complexes of four chromosomes occurred in four; one was the result of interlocking and three (Fig. 4*c–e*) were probably due to reciprocal translocation. That the latter have no relation to the segregation observed is made probable by the observation of an association of four in one nucleus of a normal sib. Such multiple complexes and failure of metaphase association are not unexpected since the parent het speltoid and its progeny all showed phenotypic evidence of hybridity.

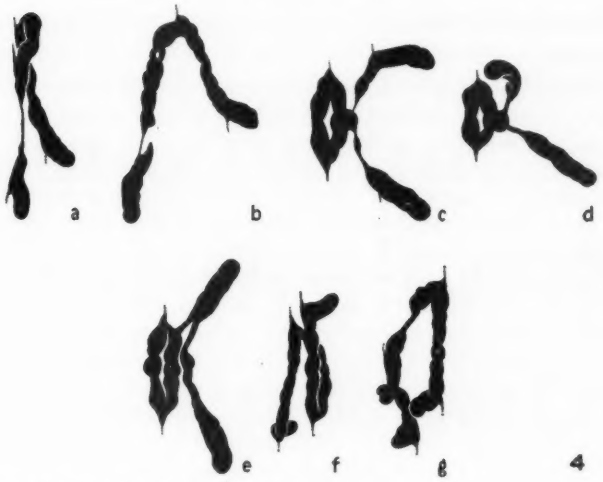
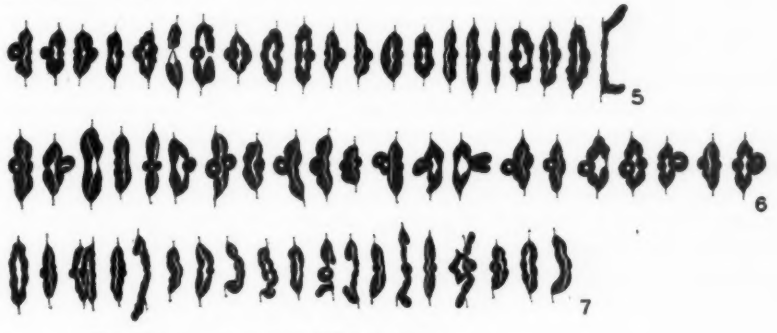


FIG. 4 *a* to *g*. Associations of four chromosomes:
4 *a* and *b*. From two normal sibs of the parent strain.
4 *c* to *e*. From a het speltoid and two of its het speltoid progeny in Substrain 1.
4 *f* and *g*. From bearded speltoid sibs of Substrain 2.

Two normals had 42 chromosomes. In 29 first metaphases they formed bivalents (Fig. 6). Once, however, as mentioned above, a complex of four chromosomes was seen but in view of their morphology these cannot be C chromosomes.



FIGS. 5 to 7. Segregates of Substrain 1:
5. Het speltoid; 20_{II} + CCd heteromorphic pair.
6. Normal sib of het speltoid; 21_{II}.
7. Bearded speltoid with 21_{II}, four of which are interlocked.

All four bearded speltoids examined had 21 bivalents (Fig. 7). The presence of two Cd chromosomes could not be proved cytologically.

(iii) The Beardless Substrain 2

(*a*) *Genetical*

The original beardless speltoid was obtained with 21 bearded speltoids as the progeny of a bearded speltoid (Table I, 14), but upon sowing the remaining seeds no further beardless speltoid was secured. (The het speltoid which initiated the bearded Substrain 1 occurred in the resowing (Table I, 15); it has already been dealt with.) Apart from the absence of beards from the one plant there were no very striking differences such as would have ensured it being classified immediately as a natural hybrid.

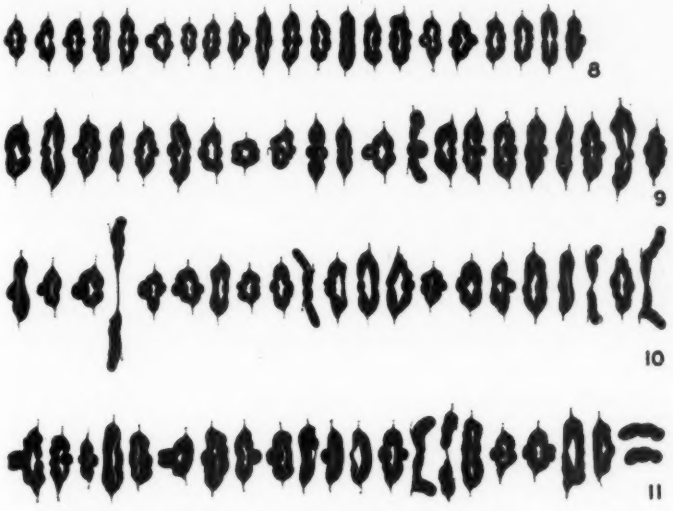
In 1929 and 1930, 85 seeds from the beardless speltoid produced a total of 75 beardless and five bearded progeny (Table I, 31 and 32). In addition to the segregation for beards here noted, the progeny showed minor differences not present in speltoids of the parental strain. According to Dr. Huskins' 1929 notes the progeny of the beardless speltoid differed as follows:— "more vigorous (taller, thicker straws, bigger heads, larger glumes, bigger grains), more brittle rachis especially at the top and most breaks are like *spelta*, with a few like *dicoccum*. There is variation in colour from light to dark brown and possibly in glume shape, which ranges from speltoid to 'speltoid tending to *spelta*' ". These beardless plants which have characteristics both of *T. spelta* and "typical" speltoids will henceforth be termed '*speltoids*'.

Four of the beardless '*speltoids*' bred true for beardlessness giving 66 beardless (Table I, 35–38), whereas three (including the original) segregated, giving 111 beardless and eight bearded '*speltoids*' (Table I, 31–34). The seven plants tested may therefore be separated into two genotypic classes not phenotypically different as regards bearding. On the other hand, the bearded descendants in general bred true in producing 160 bearded and one beardless '*speltoid*' (Table I, 48). The latter produced 27 beardless and six bearded '*speltoids*' (Table I, 42 and 43), again showing evidence of a *T. spelta* ancestry. One of the beardless '*speltoids*' in turn segregated 11 beardless and four bearded (Table I, 45), while a second bred true, producing 14 beardless progeny. Both the beardless descendants of bearded '*speltoids*' must therefore have resulted from the fertilization of a bearded-carrying ovule by a beardless-carrying pollen grain. The total number of progeny produced by the two types of beardless '*speltoids*' is thus 80 beardless from five nonsegregating beardless '*speltoids*' (Table I, 47) and 149 beardless and 18 bearded from five segregating beardless '*speltoids*' (Table I, 46). The percentages of beardless and bearded segregates are therefore 89.2 and 10.8, compared with 89.1 and 10.9 for beardless (N and H speltoid) and bearded progeny of het speltoids in the parent strain.

(*b*) *Cytological*

Thirteen plants were examined from fixations made in 1930, 1932, and 1936. They include three types, namely, the true-breeding bearded speltoid, the true-breeding beardless '*speltoid*', and the segregating beardless '*speltoid*', all being derived from original beardless '*speltoids*'.

The bearded progeny, like the bearded speltoids of the parent strain, have 42 chromosomes. In 55 nuclei these formed homomorphic bivalents (Fig. 8) but in another there were two univalents which morphologically were not Cd



FIGS. 8 to 11. Segregates of Substrain 2:

8. Bearded speltoid; 21_{II}.
9. True-breeding beardless '*speltoid*'; 21_{II}.
10. Segregating beardless '*speltoid*'; 20_{II} + heteromorphic pair.
11. Segregating beardless '*speltoid*'; the C and Cd unassociated.

chromosomes. Disjunction was normal, so that essentially only one type of gamete is expected. Genetic confirmation is supplied in that only a single beardless '*speltoid*' occurred among 161 progeny. That this resulted from natural crossing is made very probable, first, by the close proximity in the field of beardless plants and, second, by its subsequent segregation of bearded plants as mentioned above. An association of four chromosomes (Fig. 4*f* and *g*) was seen in one nucleus of each of two plants. That these are independent of the segregation observed follows from the presence of similar multiple associations in normals and het speltoids.

Both the '*speltoid*' types possess 42 chromosomes also. The true-breeding beardless type showed no heteromorphism (Fig. 9). In the segregating beardless type the complement resembled that of het speltoids in possessing an unequal pair of chromosomes. In 104 nuclei they formed a bivalent (Fig. 10); in nine nuclei they were unassociated (Fig. 11; Photomicrograph Fig. 15) and closely resembled C and Cd chromosomes.

Before concluding this Section the foregoing facts might with advantage be summarized:

(1) The bearded strain from which the substrains originated has a deficiency visible at metaphase, as does typical Series γ, but in its progeny ratio it conforms to Series α, which has not previously been shown to be determined by a deficiency.

(2) The speltoids throughout are similar to those of α strains in vigor, seed set, and germination.

(3) The het speltoid and the beardless '*speltoid*' from which Substrains 1 and 2 were derived occurred along with 101 bearded speltoids as the progeny of a bearded speltoid; the het speltoid showed indications of a *T. spelta* ancestry; the beardless '*speltoid*' segregated for bearding and for *spelta* characteristics, but the latter are difficult to distinguish from speltoids.

(4) Het speltoids also originated by natural crossing in a sib family.

(5) Among 353 speltoids descended from bearded speltoids only a single beardless '*speltoid*' occurred apart from the original one: it likewise proved heterozygous for beardlessness.

(6) Of the beardless '*speltoids*' propagated, five bred true and five segregated bearded speltoids.

(7) Both the bearded speltoids and the true-breeding beardless '*speltoids*' possessed 21 homomorphic bivalents, but the segregating beardless '*speltoids*' had a heteromorphic pair indistinguishable from that of het speltoids.

(8) Het speltoids of the parent strain segregated 10.9% bearded speltoids, and segregating beardless '*speltoids*' of Substrain 2 yielded 10.8% bearded speltoids.

IV. Crosses Involving the Beardless '*Speltoid*'

Before the cytological conditions in the beardless '*speltoid*' were fully understood a number of crosses were made by Dr. Huskins to throw light on its mode of origin and to further knowledge of the interrelationships of the various factors contributing to the speltoid-bearding complex.

The types used and the appearance of the F_1 with respect to bearding and glume shape are as follows:

(*a*) *T. spelta* beardless × beardless '*speltoid*' . . . 4 beardless *spelta* type.

(*b*) Beardless '*speltoid*' × α beardless speltoid . . . 6 beardless speltoids.

(*c*) Beardless '*speltoid*' × bearded *T. dicoccum* . . . 3 beardless + 2 bearded.

(*d*) Beardless '*speltoid*' × α bearded speltoid . . . 9 beardless speltoids.

(*e*) Beardless '*speltoid*' × *T. vulgare leucospermum* . . . 5 beardless het speltoids.

(*a*) In the F_2, although segregation occurred for glume color and pubescence of the base of the rachilla, all the 254 progeny were completely beardless and had uniformly *spelta*-shape glumes. It is therefore apparent only that the

PLATE I

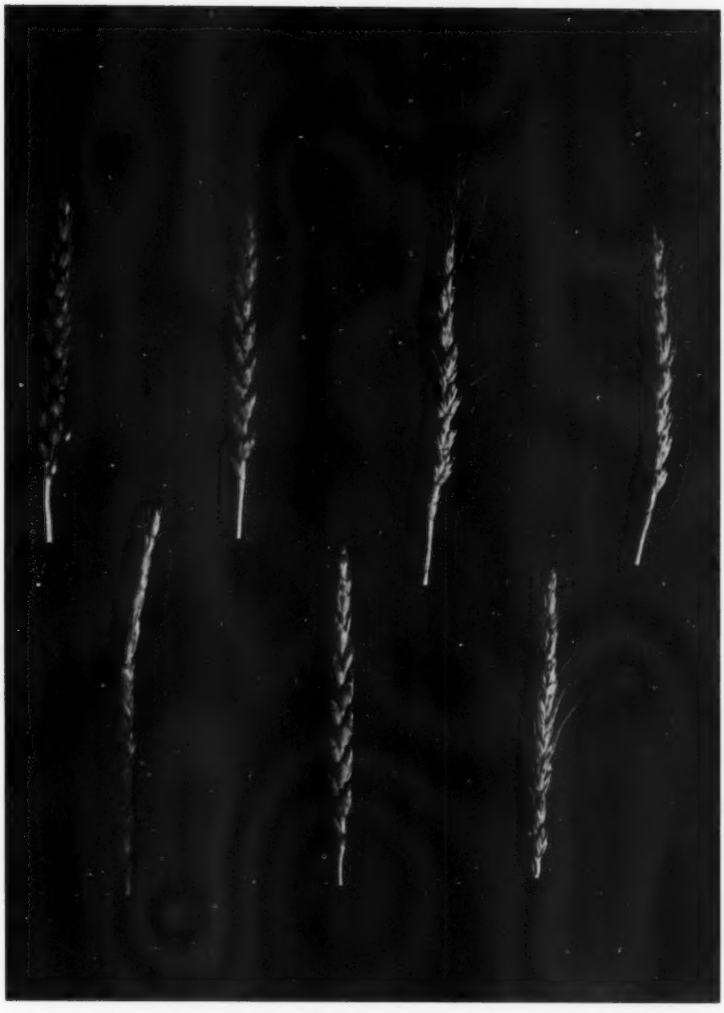
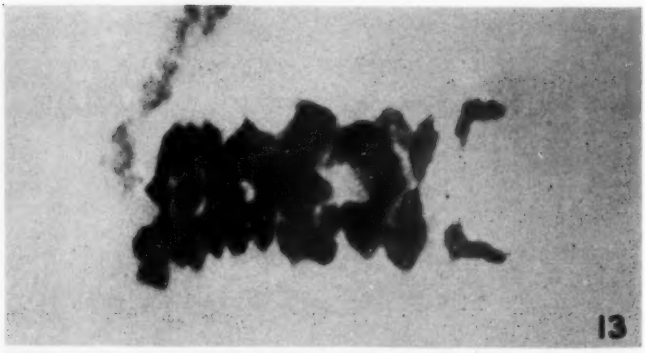
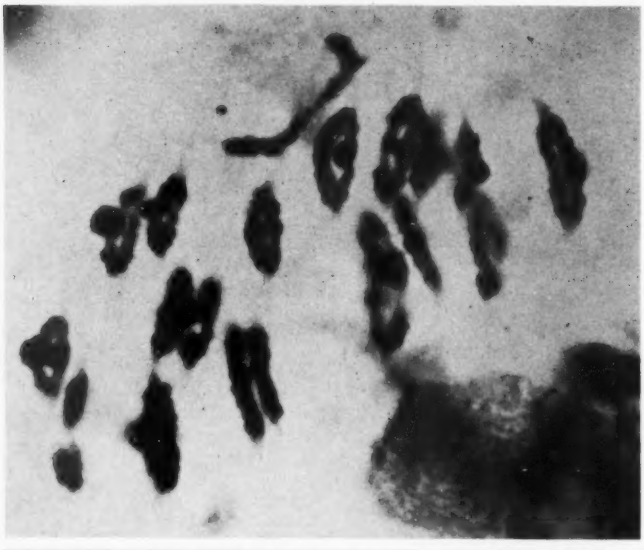
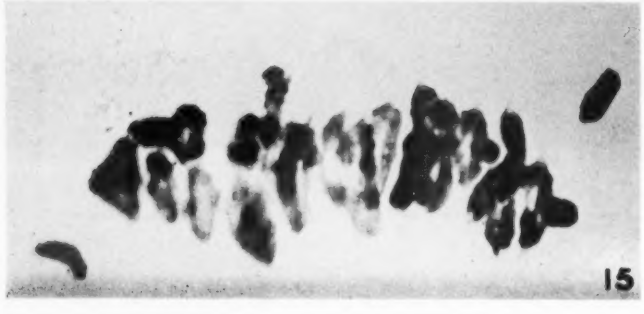


FIG. 12. Top row: normal, het speltoid, and bearded speltoid of the parental strain, and the bearded speltoid parent of Substrain 2. Bottom row: true-breeding beardless '*speltoid*', segregating beardless '*speltoid*', and true-breeding bearded speltoid of Substrain 2.

PLATE II







male gametes that functioned, like those of the female parent, carried beard inhibitors and *spelta*-glume factors. It is concluded that the male parent was "homozygous".

(*b*) The male parent used in this cross was obtained from Mr. A. E. Watkins, Cambridge, England. It was a segregate from the cross *T. vulgare* × *T. turgidum* and produced Series α beardless het speltoids when crossed with *T. vulgare*. All 220 F_2 plants, like those of the F_1, were uniformly beardless. They showed segregation for a number of minor characters, but no detailed analysis was attempted. Since all the descendants received beard inhibitors from both parents, it is evident that the female parent belonged to the non-segregating type.

(*c*) The male parent in this cross was a bearded tetraploid: the F_1 plants consequently had 35 chromosomes. Partly owing to this, segregation in the F_2 and F_3 was extremely complex, forming a more or less complete series between the two parents. However, from the occurrence of both beardless and bearded plants in the F_1, it is evident that the beardless parent must have been heterozygous for the beard inhibitors.

(*d*) The bearded speltoid male parent, like the earlier mentioned beardless speltoid, came from one of Mr. Watkins' Series α lines. All nine F_1 plants were uniformly beardless, making it probable that only ovules carrying beard inhibitors were produced by the female parent. That the F_1 plants are heterozygous is shown by the F_2 and F_3 segregation (Table II).

TABLE II

INHERITANCE OF BEARDING IN THE F_2 AND F_3 GENERATIONS OF THE CROSS BEARDLESS '*speltoid*' × α BEARDED SPELTOID

Parent	Generation	Number of families	Speltoids		Total
			B'less	B'dd	
1. Bb Sp F_1	F_2	4	126	37	163
2. Bb Sp F_2	F_3	9	145	36	181
3. BB Sp F_2	F_3	5	65	0	65
4. bb Sp F_2	F_3	3	1*	34	35
5. Total for seg. Bb		13	271	73	344

* *Natural cross.*

Photomicrographs of acetocarmine smear preparations of pollen mother cells

FIG. 13. Metaphase I, het speltoid of parent strain. The C and Cd chromosomes associated as a heteromorphic bivalent.

FIG. 14. Metaphase I showing the heteromorphic CCd bivalent in a het speltoid from a natural hybrid of Substrain 1.

FIG. 15. Metaphase I, segregating beardless '*speltoid*'. The Cd of the parental strain and the C of a *T. spelta* derivative are unassociated.

The mode of segregation in cross (*d*) may be expressed diagrammatically as follows:

$$(BB)\ .\ .\ C^sC^s \quad x \quad C^\alpha C^\alpha\ .\ .\ (bb)$$

$$C^sC^\alpha\ .\ .\ (Bb)$$

$$(BB)\ .\ .\ C^sC^s \quad C^sC^\alpha \quad C^\alpha C^\alpha\ .\ .\ (bb)$$

where C^s represents the C chromosome introduced originally from the beardless *T. spelta* derivative, and C^α represents the C chromosome contributed by the Series α bearded speltoid.

(*e*) In the last cross the male parent was *T. vulgare leucospermum* Körn, with the phenotype "beardless normal". The introduction of a complete set of beard inhibitors and *vulgare* genes resulted in the production of beardless het speltoid F_1 plants. The progeny of this cross has already been analyzed in detail by Armstrong (1) who reported on the mode of inheritance of 13 alternative pairs of factors introduced by the respective parents. The present consideration will therefore be limited to the speltoid-bearding complex.

The summarized F_2 and F_3 results (Table III) demonstrate conclusively that the two F_1 plants analyzed differ in their genic constitution, the one producing bearded speltoids, the other beardless speltoids, owing to their possession

TABLE III

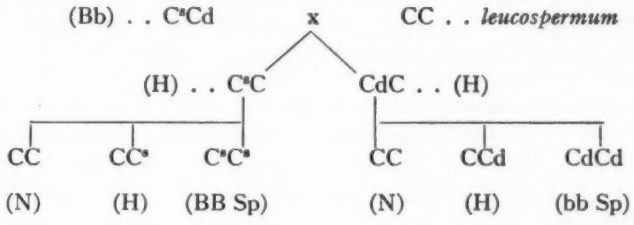
INHERITANCE OF GLUMES AND BEARDING IN THE F_2 AND F_3 GENERATIONS OF THE CROSS BEARDLESS '*speltoid*' × *T. vulgare leucospermum*

Parent	Generation	Number of families	N	H	B'less	B'dd	Total
1. Het Sp F_1 plant	F_2	1	11	24	0	4	39
2. Het Sps ex F_2	F_2	8	38	69	0	14	121
3. Total		9	49	93	0	18	160
4. Het Sp F_1 plant	F_2	1	9	22	12	0	43
5. Het Sps ex F_2	F_3	5	26	38	24	0	88
6. Total		6	35	60	36	0	131

of Cd and C^s chromosomes respectively. This segregational difference in the F_2 and F_3 not only places the female parent in the segregating category but, as will be shown, provides a further means of estimating the degree to which certation operates against pollen carrying the Cd chromosome of the female parent.

When the speltoid segregates are all beardless, they demonstrate the presence in duplicate of the C^s chromosome which originated the beardless Substrain 2. When they are all bearded, they prove the presence of the

alternative Cd chromosome in duplicate, *i.e.*, to the exclusion of the C chromosome introduced from *leucospermum*. This may be expressed diagrammatically as follows:



where C represents the C chromosome of *leucospermum*, C^s the C chromosome of a beardless *T. spelta* derivative, and Cd the segmentally deficient C chromosome of $\alpha 3$.

It is obvious that segregating beardless speltoids are no longer possible in subsequent generations of either line.

V. Discussion

The Parent Strain

The frequency (237 : 317 : 68) with which normals, het speltoids, and bearded speltoids are segregated by het speltoids of the parent strain obviously deviates greatly from both a 1 : 2 : 1 and the γ expectation, but it gives a reasonable fit to an established α ratio. To test whether the deviation is statistically significant, χ^2 tests of goodness of fit have been applied. The observed figures have been fitted (Table IV, 3 and 4) against those determined for two strains, $\alpha 1$ (55 : 95 : 32) and $\gamma 2$ (272 : 304 : 10), the cytological classification of which has been previously established (11). The present strain differs significantly from $\gamma 2$ but may be assigned to the α group.

As was stated earlier, the parental $\alpha 3$ strain was obtained from Åkerman's speltoid strain which originated from Brown Schlanstedt × "0715" (See 15, Table I). The progeny ratios of the two should therefore be similar, and it is shown in Table IV, 14 that they are in fact statistically identical.

Het speltoids with a C chromosome deficiency long enough to be visible at metaphase have in all other cases given γ ratios. In this strain the ratio indicates that the mutant pollen functions almost as well as in typical α. Deviations from a 1 : 2 : 1 ratio towards 1 : 1 : 0 are determined primarily by certation, the mutant pollen being at a disadvantage in competition with normal pollen. In general, then, unless there is marked differential mortality (which in cultures of spring wheat, at least, appears to be negligible (11), in those strains in which essentially only two types of gametes are produced and these in equal numbers, the relative functioning capacity of normal and mutant pollen may be determined from the observed progeny ratio. This may be expressed as: normal = N + H − Sp : mutant = 2 Sp. Thus

in an ideal monofactorial ratio the proportion, 25 + 50 − 25 : 2 × 25, is, of course, 1 : 1 — certation is absent; in $\alpha 1$ (55 : 95 : 32) certation occurs as 1.8 : 1; and in $\alpha 3$ (237 : 317 : 68) to the extent of 3.6 : 1.

In typical γ strains, where the extent of the deficiency from the mutated C chromosome is sufficiently great to interfere with its pairing with the unaltered C chromosome, normal segregation at meiosis is disturbed and 20-chromosome gametes are formed. As pollen these play little if any part in distorting the progeny ratio; as ovules they increase the proportion of het speltoid relative to normal segregates—that is, they act in opposition to the certation factor of the male side. Theoretically, then, any excess of het speltoids over the total of normal plus speltoid progeny may be considered to comprise β het speltoids.

In $\gamma 2$ a relatively high frequency of nonconjugation occurred in pollen mother cells, and 14 % of the het speltoids proved, either cytologically or by breeding tests, to be β Series (11). Its progeny ratio may therefore be expressed as 272N : 262γH : 42βH : 10 Sp. Because of the inclusion of the β het speltoids, it is not possible to arrive at a precise estimate of the degree of certation, for they point to the possibility that some of the speltoids may have the constitution 40 + Cd, and such speltoids can not be distinguished phenotypically from those with 40 + CdCd. It is possible therefore to arrive at only a rough approximation of the degree of certation operating in typical γ strains. For comparative purposes, however, this is sufficiently close, for it can not be far from 28.3 : 1 in the case of $\gamma 2$.

The three values so obtained, 1.8, 3.6, and 28.3 to unity, for $\alpha 1$, $\alpha 3$, and $\gamma 2$ respectively, make it quite clear that differences in certation are the major factors differentiating the three progeny ratios and that the mutant pollen of $\alpha 3$ is considerably closer in innate capacity to function to that of $\alpha 1$ than to that of $\gamma 2$.

Although segregation in the parental strain is thus clearly Series α and therefore a distortion of 1 : 2 : 1, Uchikawa's (13) strains, derived from two of the parental D_1 het speltoids, gave progeny which, summated over four generations, fitted fairly well a 1 : 2 : 1 expectation. His grouped totals, 230N : 424 H : 179 Sp, are therefore markedly different statistically from the 237 N : 317 H : 68 Sp obtained herein. It is certain, then, that his strains are genetically distinct from $\alpha 3$. If his two strains were each derived from single plants of his first generation (as the numbers of progeny suggest), it may well be that these two plants came from hybrid seeds (originating by natural crossing on the heads sent to him in the same manner as the beardless '*speltoid*' considered here) and that he especially selected them for their vigor and marked "het speltoid" phenotype. This possibility is considerably strengthened in that Uchikawa's photographs and measurements show that his two derived strains are phenotypically distinct, though similar in genetic behavior. A considerable amount of natural crossing with derivatives of a *T. vulgare* – *T. spelta* cross occurred in the year in which these heads matured, and there are signs in his photographs of a *T. spelta* ancestry. In the absence of information

to the contrary, it seems best, therefore, to attribute the difference between Uchikawa's and the writer's results to the introduction by natural crossing of a *spelta* gamete which suffered little from certation.

If Uchikawa's material is considered to be CC^s (normal C of *vulgare* and "normal" C of *spelta*) with a certation factor of *ca.* 1.3 : 1, whereas $\alpha 3$ herein is CCd with a certation factor of 3.6 : 1, their segregational differences are readily reconciled (see also later).

Origin and Behavior of Substrains

Series γ bearded speltoids are produced by combination of two Cd gametes. That is, the hypostatic bearding factors are capable of expression in the absence, through segmental deficiency, of the epistatic beard inhibitors from the Cd chromosome. When, therefore, the epistatic factors have been lost from both C chromosomes, as in speltoids of the parental strain, the origin of a beardless speltoid from a bearded speltoid necessitates the introduction of a chromosome carrying inhibitors of the beard factors but at the same time devoid of the inhibitor that determines normal glumes (since otherwise a beardless het speltoid would result, as in fact one has—Substrain 1).

Now although the factors epistatic to bearding and speltoid are completely linked in het speltoids arising in one step, the introduction of a C chromosome lacking the beard inhibitor alone is nevertheless possible in this case. In 1927 there was growing in an adjacent plot a family which was classified by Huskins (2) as being contaminated with *T. spelta* genes and which was later shown to be the F_2 from a natural hybrid between a speltoid strain of *T. vulgare* and a derivative of a *T. spelta* cross (11). From the segregation observed, the *T. spelta* ancestor must have been beardless; it, therefore, has the beard inhibitors but not the factor which inhibits the genes for *spelta* or speltoid characteristics. The cross-fertilization of a parental strain ovule lacking beard inhibitors (Cd) by a pollen grain carrying them (C^s) would account for the occurrence of a beardless zygote. The segregation in later generations for glume shape in the direction of *T. spelta* makes such a mode of origin highly probable, for it is quite conceivable that along with the C^s chromosome others may have been introduced bearing factors modifying glume shape. It is important to note, however, that the putative male gamete came from an F_2 or later generation, not from a pure *T. spelta* plant. This accounts for the absence of clear cut segregation for other factors in the progeny of the original beardless plant.

The phenotype of the het speltoid that arose at the same time (Substrain 1) and was classified as a "possible beardless speltoid" when first examined readily suggests that it likewise arose through hybridization with a sib of the plant concerned in the origin of the beardless '*speltoid*'. Here, however, the male gamete must have differed in carrying a *T. vulgare* C chromosome having the normal factors for the beardless-*vulgare* complex. In addition other chromosomes must have borne modifying factors for *spelta* rather than speltoid glumes.

If the beardless '*speltoid*' originated as postulated, it should have been heterozygous for the beard inhibitors and should, if the two types of male gametes were of equal functioning capacity, have been capable of segregating three beardless to one bearded speltoid. That it was heterozygous is clear from its segregation, but there was a marked shortage of bearded speltoids. It has already been shown for het speltoids of $\alpha 3$ that the Cd pollen grains function only once to every 3.6 C. There appears to be no a priori reason for expecting the C^s chromosome of *T. spelta* to differ from the C of *T. vulgare* with respect to its effect on certation. The differential acting against Cd pollen in the segregating beardless '*speltoid*' should thus approximate that operating in the parental strain het speltoids. Obviously it does. Computed from the grouped progeny of five segregating beardless '*speltoids*', the relative frequency with which the two types function is again 3.6 C^s : 1 Cd (Table V, 5). Expressed differently, the ratio of normals and het speltoids to bearded speltoids should be, as it is (Table IV, 5), like that for beardless to bearded speltoids. Finally, since the beard inhibitors are epistatic, the beardless category must be composed of two types, the one heterozygous and the other homozygous. These have been realized in that five segregating and five non-segregating beardless have been identified by progeny tests. Further confirmatory evidence is forthcoming from the analysis of certain of the crosses involving the beardless '*speltoid*'.

Analysis of Crosses

The first three crosses provide evidence only as to whether the beardless '*speltoid*' parent was heterozygous or homozygous for the beard inhibitors. In cross (*d*) the F_1 plants differed from Substrain 2 segregating beardless '*speltoids*' only in the greater frequency with which they gave rise to bearded speltoids. This confirms the conclusion already reached as to the cause underlying the deficiency of bearded speltoids in the original strain. Fitting the segregation ratio of the beardless Substrain 2 against either a monofactorial or an established α ratio gives *P* values (Table IV, 6 and 7) which show that only the deviation from a monofactorial ratio is significant. In contrast, the combined F_2 and F_3 segregating beardless speltoids give a ratio which is statistically different from neither (Table IV, 8 and 9). The percentages of bearded speltoids produced in the two cases are 10.8 and 21.2 respectively. Since the maternal genotype is the same in both, the difference must result solely from differences introduced by the male parent in the cross, that is, the reduction in certation attributable to the C^α chromosome relative to the original Cd chromosome (Table V, 7).

It is of particular interest to note also that the ratio from this cross (271 : 73) is statistically identical to the segregation observed by Uchikawa (13) when his normals and het speltoids are grouped (Table IV, 15). This is strong evidence that Uchikawa's strains did in fact originate by natural crossing and are therefore genetically distinct from the parental $\alpha 3$ strain.

As a result of cross (*e*), cytological conditions essentially equivalent to those in $\alpha 3$ have been restored in the F_1 het speltoid which segregated the

TABLE IV

SIGNIFICANCE OF OBSERVED PROGENY RATIOS

Test	Observed ratio	Table	Tested against	Table, series, or reference	χ^2	P
1	76 : 78 : 16	I, 21	237 : 317 : 68	I, 6	2.456	<.3
2	25 : 29 : 7	I, 30	76 : 78 : 16	I, 16	0.361	<.9
3	237 : 317 : 68	I, 6	55 : 95 : 32	α 1	7.459	>.02
4	237 : 317 : 68	I, 6	272 : 304 : 10	γ 2	44.771	<.01
5	149 : 18	I, 46	554 : 68	I, 6	0.004	.95
6	149 : 18	I, 46	3 : 1		18.017	<.01
7	149 : 18	I, 6	150 : 32	α 1	3.285	>.05
8	271 : 73	II, 45	3 : 1		2.619	>.1
9	271 : 73	II, 5	150 : 32	α 1	0.987	>.3
10	49 : 93 : 18	III, 3	1 : 2 : 1		16.237	<.01
11	49 : 93 : 18	III, 3	237 : 317 : 68	I, 6	3.203	>.2.
12	35 : 60 : 36	III, 6	1 : 2 : 1		0.939	<.7
13	35 : 60 : 36	III, 6	230 : 424 : 179	U '41	2.442	<.3
14	237 : 317 : 68	I, 6	4910 : 6087 : 1301	A&M '48	0.821	<.7
15	271 : 73	II, 5	654 : 179	U '41	0.097	>.7

bearded speltoids, for this plant has the constitution CCd. The former chromosome, being derived from a normal variety of *T. vulgare*, should confer no handicap on male gametes bearing it. Consequently, the considerable deviation from a 1 : 2 : 1 ratio (Table IV, 10) must be referable directly to the innate low competitive value of Cd pollen (Table V, 8). Because of this the parental strain and the bearded speltoid-producing hybrid in this cross should exhibit comparable segregation. This they do (Table IV, 11). On the other hand, the segregation in the six beardless families fits a monofactorial ratio (Table IV, 12) as is expected in view of their mode of origin, for C^s and C-containing pollen should, as argued earlier, be of approximately equal functioning capacity (Table V, 9; cf. also Table IV, 5).

TABLE V

ESTIMATES OF CERTATION FACTORS BASED ON THE FORMULA N + H − SP : 2 SP

	Type	Progeny ratio	Certation factor	Reference
1.	α1	55 : 95 : 32	1.8 : 1	(11)
2.	α3	237 : 317 : 68	3.6 : 1	Herein
3.	γ2	272 : 304 : 10	28.3 : 1	(11)
4.	α	230 : 424 : 179	1.3 : 1	(13)
5.	Substrain 2	149 B'less : 18 B'dd	3.6 : 1	Herein
6.	Substrain 1	76 : 78 : 16	4.0 : 1	Herein
7.	Cross (*d*)	271 B'less : 73 B'dd	1.4 : 1	Herein
8.	Cross (*e*)	49 : 93 : 18	3.4 : 1	Herein
9.	Cross (*e*)	35 : 60 : 36	0.8 : 1	Herein

The results of these crosses go far in substantiating the earlier conclusions regarding the origin of the beardless '*speltoid*' Substrain 2, and the progeny ratios of the het speltoids and segregating beardless '*speltoids*'. They clearly show that the beardless substrain arose through crossing with a plant which itself was a hybrid with beardless *T. spelta* (cross *a*) and that the beardless '*speltoid*' plants are of two types, one homozygous and the other heterozygous for the epistatic beard inhibitors (crosses *b* – *e*). They further demonstrate (crosses *d* and *e*) that the normal C of het speltoids differs from the C^s of segregating beardless '*speltoids*' in carrying factors epistatic to the speltoid factors borne elsewhere.

Neither het speltoids nor segregating beardless '*speltoids*' give a good monofactorial ratio because of the segmental deficiency which imposes extreme certation on Cd pollen through competition with either C or C^s pollen. It is to be expected though that C^s pollen would have little advantage when in competition with mutant pollen of a "good" Series α and none relative to normal *T. vulgare* pollen. These expectations were fully realized in the crosses, for when C^s pollen competed with introduced C^a pollen its advantage was considerably reduced (observed 21% bb speltoids; expected 25% in cross *d*), and when competition was provided by *leucospermum* pollen its advantage was almost completely nullified (35N : 36 Sp in cross *e*). On the other hand, Cd pollen suffered in competition with *leucospermum* pollen to almost the same extent as when competition was provided by normal pollen in the parental strain (49N : 18 Sp in cross *e*, and Table IV, 11). Hence the deviations from a monofactorial ratio in the parent strain and the substrains are again clearly shown to be due primarily to certation; this must be conditioned by the visible deficiency from the Cd chromosome in the parental $\alpha 3$ strain.

It has already been shown that the characteristic γ segregation is likewise determined largely by certation superimposed on two types of ovules produced in near equality, and that nonconjugation of C and Cd, resulting from the deficiency in the latter, leads to the production of 20-chromosome ovules capable of giving rise to β het speltoids. In three γ strains studied by Smith, Huskins, and Sander (11), the deficiency from the mutant C chromosome was, as in the present case, located interstitially in the distal half of the long arm, while in a fourth, $\gamma 4$, it consisted of the whole of the long arm. Owing to the deficiency, the C and Cd chromosomes were unassociated at metaphase in some of the pollen mother cells. While it is recognized that metaphase association is greatly influenced by extrinsic factors, since changes in linear homology obviously interfere with pairing, it is to be expected that the larger the interstitial deficiency, the greater the frequency of univalents in heterozygotes. Measurements of the relative lengths of the C and mutated C chromosomes show that the percentage deficiencies from the latter in α and γ strains are roughly proportional to the frequency of nonassociation (Table VI). In $\gamma 4$, the strain in which the entire long arm of C is missing, the frequency of univalents is unexpectedly low. This may be related indirectly to the fact

TABLE VI

CORRELATED PROPERTIES OF VARIOUS SPELTOID STRAINS

Series and strain	Mean length of mutant C	% Conjug.	Proportion N : Sp progeny	% β hets*	Coefficient of certation
β1 to 4	0.0	0.0	31 : 1	100.0	.002
γ4	15.0	94.0	230 : 1	5.9**	.004
γ2	73.9±1.46	86.4	27 : 1	13.9	.032
γ1	75.4±0.87	88.0	12 : 1	9.1	.073
α3	79.6±0.84	93.3	3.5 : 1	0.0	.279
α1	?	*ca.* 100	1.7 : 1	0.0	.559

* *This percentage is based upon the number of het speltoids among the total het speltoids that were β either genetically or cytologically.*

** *One of two supposedly γ het speltoids supplied to Uchikawa proved to be β (see Footnote 2, p. 5 in (13).*

that there is no interference in the orderly progression of pairing between the centromere and the end of the arm (10). It is suggested also by the frequency of pairing of the two Cd chromosomes in other γ speltoids being higher than that of the C with Cd in their corresponding het speltoids (unpublished data). With the exception of γ4 a rough correlation might therefore be expected between the extent of the deficiency from the mutated C chromosome, the percentage conjugation between it and the C, the proportion of normal to speltoid progeny, the percentage of β segregates among the het speltoid progeny of γ het speltoids, and the coefficient of certation, i.e., the frequency of functioning of mutant pollen in relation to its frequency of formation. That this expectation is reasonably fulfilled is shown in Table VI, where even the γ4 strain is shown to fit in some respects. Notably, there is good agreement for γ4 in percentage conjugation and the rarity of β het speltoid progeny; also in the length lost from the mutant C chromosome and the coefficient of certation. The remarkably high proportion of normal to speltoid progeny is, of course, a dual result of it having an extremely high certation coefficient for a γ strain and the fact that the frequency of normal progeny approaches 50%. On the other hand, the apparent conflict in the proportion of normal to speltoid segregates in β and γ2 results from the markedly different frequencies with which mutant gametes are both formed and function.

Where progeny ratios deviate markedly from 1 : 2 : 1 or 1 : 1 : 0 in that het speltoids form more than 50% of the progeny and where differential mortality can be ruled out, it is plausible to argue that the excess of het speltoids is due to the formation of 20-chromosome ovules. The percentage of hets that may be suspected of being β may be deduced from the following formula, $\frac{H - (N + Sp) \times 100}{H}$. Data given by Åkerman and Mac Key (15), by Smith, Huskins, and Sander (11), and by Uchikawa (13) fit the hypothesis fairly well (Table VII) only in those strains where sufficient progeny

TABLE VII

OBSERVED AND EXPECTED FREQUENCY OF β HET SPELTOIDS IN HET PROGENY OF α AND γ HET SPELTOIDS (VARIOUS AUTHORS)

Reference	Series	Per cent expected	Per cent observed	Number tested
(15) Table I	α	0.0	0.0	175
(15) Table IV	γ	0.5	3.5	145
(15) Table VI	γ	4.3	3.8	160
(15) Table IX	γ	0.0	0.0	31
(15) Table X	γ	0.7	0.0	38
(11) Table IX	$\alpha 1$	8.4	0.0	7
Herein	$\alpha 3$	3.8	0.0	20
(11) Table VI	$\gamma 1$	15.9	9.1	14
(11) Table VII	$\gamma 2$	7.2	13.9	36
(11) Table VIII	$\gamma 4$	14.8	5.9*	15
(13) Table 4*a*	α	0.9	0.0	Not given
(13) Table 4*b*	α	6.2	0.0	Not given

* *See footnote to Table VI herein concerning the β het speltoid in this strain.*

tests have been made. In others, where they fit poorly, there is always the possibility that β het speltoids have been neglected in selecting plants to provide future generations because as a rule they are somewhat less vigorous.

While agreeing that het speltoids of Series β and γ result respectively from whole and part chromosome deficiencies, Uchikawa (13) states "it appears most probable that the A Series speltoid mutation is caused by mutation of genes, whose loci are on the C-chromosome". He assumes (without evidence) that the genes *sp* and *ar* (for speltoid glumes and bearding respectively) are tightly linked and carried on the B chromosome—they are hypostatic to two inhibitors located on the C chromosome. He continues "If there occur simultaneously mutation of these (C chromosome) genes to their recessive state (i_1 , i_2), namely, the (bearded) speltoid character, will take place viz.:

$A^+ B^{sp}, {}^{ar} C^+ \ldots$ *vulgare* ———— $A^+ B^{sp}, {}^{ar} C^{i}_{1}, {}^{i}_{2} \ldots$ speltoid".

In this he completely disregards the contrary evidence of Nilsson-Ehle (6) concerning the looseness of linkage between the inhibitors on the C chromosome, and also fails to account for the absence of part mutants from the progeny of his het speltoids.

Despite the fact that Strain $\alpha 3$, Åkerman and Mac Key's (15) speltoid strain "from Brown Schlanstedt × 0715", and Uchikawa's material have a common origin, the progeny ratios of the first two alone are homogeneous (Table IV, 14); unlike Uchikawa's ratio they both fail even remotely to fit a 1 : 2 : 1 expectation. Neither Åkerman and Mac Key nor Uchikawa have detected the presence of a CCd bivalent in het speltoids of their materials. The former merely state that "both the het speltoid and the hom speltoid have 42 chromosomes. The chromosome behaviour was normal . . ." Since their material is identical in origin and genetic behavior with $\alpha 3$ herein, it may be that they missed the heteromorphism described here in $\alpha 3$. Uchikawa,

on the other hand, found association of chromosomes in fours in about 0.3% of the nuclei of normal, het speltoids, and speltoids; similar multiple complexes have been observed sporadically in the parental $\alpha 3$ and the two substrains. These are most logically attributed to hybridization. For the several reasons given already, it seems most reasonable to explain Uchikawa's failure to observe the presence of a CCd pair in his het speltoids by the fact that the Cd has been replaced there by a C^s chromosome which is little or no different in size from the normal C.

Little can be added from this study to the discussion of the nature of the speltoid mutation given earlier in this series of papers. It is perfectly evident that there is no clear dividing line differentiating γ from α ratios, for theoretically it is possible to have a continuous series of ratios extending all the way from 1 : 1 : 0 to 1 : 2 : 1. In the absence of differential mortality the former is probably never realized in practice since it requires certation to reach its absolute limit. This limit is to be expected only with the grossest segmental deficiency from the mutated C chromosome; so gross in fact that, through nonconjugation, it would inevitably result in the production of a considerable proportion of 20-chromosome ovules, and thus cause an appreciable increase in the number of het speltoids relative to normals. On the other hand, since the "total mutation" from beardless normal to bearded speltoid affects a region of the chromosome between which 27 to 39% crossing over has been demonstrated (6, 14), it is apparent that the mutant male gametes of even the "best" α bearded speltoid strains will be subject to some degree of certation and a consequent deficiency of speltoid segregates. Part mutants, such as beardless speltoid strains, should and do give a much closer fit to a 1 : 2 : 1 expectation (Table IV, 1; Table III, 6; and $\alpha 2$ in (11)).

The genetic evidence alone has earlier been regarded (8, 9, and 11) as making it highly probable that a deficiency is involved in Series α as in γ. Cytologically it has been demonstrated with complete certainty to date only in the parental α strain reported on herein. That a comparable deficiency is not demonstrable in other α strains points to it being of very restricted length and this, at first sight, is difficult to reconcile with its length when measured genetically. It is difficult to escape the conclusion that the B—27+—k region is relatively short but is located in a segment of the chromosome which is particularly liable to crossing over. Should this be so, the variable frequency with which the C and Cd chromosomes of different γ strains fail to conjugate is readily understandable: they obviously differ in the length of the deficient segment in which the B—27+—k region is included.

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Page 481. In the last line of the page ther conifers should read other conifers.

Page 484. In the last line of the captions for Text-fig 1, Cooper River should read Copper River.

Page 505. In the last line above Table XI noted than should read noted that.

Page 517. In the headnote for Table XX, (Basic 47 acres) should read (Basis 47 acres).

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